PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/86, 15/12, C07K 14/705, A61K 48/00

A2

(11) International Publication Number: **WO 98/15638**

(43) International Publication Date: 16 April 1998 (16.04.98)

(21) International Application Number: PCT/US97/17948

(22) International Filing Date: 6 October 1997 (06.10.97)

(30) Priority Data:
60/027,891 6 October 1996 (06.10.96) US

(71) Applicant: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 1345 Avenue of the Americas, New York, NY 10105 (US).

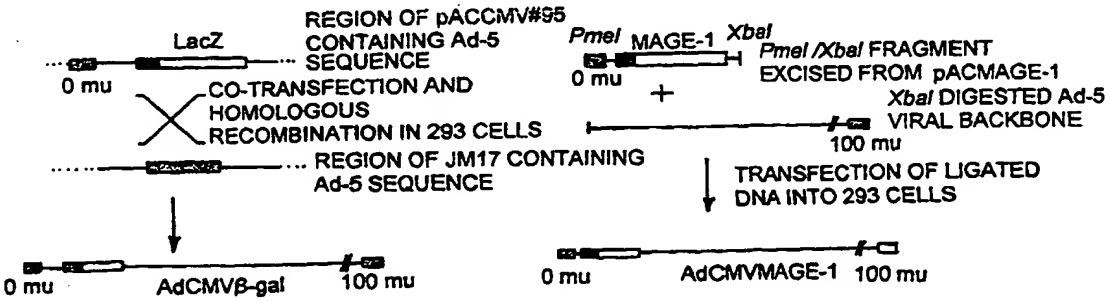
(72) Inventors: REED, Darryl, S.; Chemin des Boveresses 155, CH–1066 Epalinges (CH). ROMERO, Pedro; Chemin des Boveresses 155, CH–1066 Epalinges (CH). RIMOLDI, Donata; Chemin des Boveresses 155, CH–1066 Epalinges (CH). CERROTTINI, Jean–Charles; Chemin des Boveresses 155, CH–1066 Epalinges (CH). JONGENEEL, C., Victor; Chemin des Boveresses 155, CH–1066 Epalinges (CH).

(74) Agent: VAN AMSTERDAM, John; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

(81) Designated States: AU, CA, CN, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: REPLICATION–DEFECTIVE ADENOVIRUSES FOR CANCER IMMUNOTHERAPY



(57) Abstract

The invention provides compositions and methods for using replication–deflective adenoviruses and adenovirus genomes to introduce nucleic acids encoding tumor rejection antigen precursors into cells. The compositions and methods are useful for, *inter alia*, inducing or enhancing by *in vivo* or *ex vivo* means the immune response of a subject to tumor rejection antigens, preparation of cytolytic T lymphocytes and analysis of tumor rejection antigen precursor processing.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

REPLICATION-DEFECTIVE ADENOVIRUSES FOR CANCER IMMUNOTHERAPY

Field of the Invention

This invention relates to replication-defective adenovirus genomes which contain nucleic acid molecules encoding tumor rejection antigen precursors, which are expressed preferentially in tumors. The replication-defective adenovirus genomes and adenoviruses containing the replication-defective adenovirus genomes are useful in, *inter alia*, diagnostic and therapeutic contexts.

Background of the Invention

The phenotypic changes which distinguish a tumor cell from its normal counterpart are often the result of one or more changes to the genome of the cell. The genes which are expressed in tumor cells, but not in normal counterparts, can be termed "tumor specific" genes. These tumor specific genes are markers for the tumor phenotype. The expression of tumor specific genes can also be an essential event in the process of tumorigenesis.

Typically, the host recognizes as foreign the tumor specific genes which are not expressed in normal non-tumorigenic cells. Thus, the expression of tumor specific genes can provoke an immune response against the tumor cells by the host. Tumor specific genes can also be expressed in normal cells within certain tissues without provoking an immune response. In such tissues, expression of the gene and/or presentation of an ordinarily immunologically recognizable fragment of the protein product on the cell surface may not provoke an immune response because the immune system does not "see" the cells inside these immunologically privileged tissues. Examples of immunologically privileged tissues include brain and testis

The discovery of tumor specific expression of a gene provides a means of identifying a cell as a tumor cell. Diagnostic compounds can be based on the tumor specific gene, and used to determine the presence and location of tumor cells. Further, when the tumor specific gene is essential for an aspect of the tumor phenotype (e.g., unregulated growth or metastasis), the tumor specific gene can be used to provide therapeutics such as antisense nucleic acids which can reduce or substantially eliminate expression of that gene, thereby reducing or substantially eliminating the phenotypic aspect which depends on the expression of the particular tumor specific gene.

As previously noted, the polypeptide products of tumor specific genes can be the targets

for host immune surveillance and provoke selection and expansion of one or more clones of cytotoxic T lymphocytes specific for the tumor specific gene product. Examples of this phenomenon include proteins and fragments thereof encoded by the MAGE family of genes, the tyrosinase gene, the Melan-A gene, the BAGE gene, the GAGE gene, the RAGE family of genes, and the brain glycogen phosphorylase gene as are detailed below. Thus, tumor specific expression of genes suggests that such genes can encode proteins which will be recognized by the immune system as foreign and thus provide a target for tumor rejection. Such genes encode "tumor rejection antigen precursors", or TRAPs, which may be used to generate therapeutics for enhancement of the immune system response to tumors expressing such genes and proteins.

The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T cell response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLA"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction of T cells and complexes of HLA/peptide is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. The mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, *Science* 257: 880, 1992; Fremont et al., *Science* 257: 919, 1992; Matsumura et al., *Science* 257: 927, 1992; Latron et al., *Science* 257: 964, 1992.

The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., *Immunogenetics* 35: 145,1992; van

der Bruggen et al., *Science* 254: 1643,1991, for further information on this family of genes. Also, see U.S. patent application serial number 807,043, filed December 12, 1991, now U.S. Patent No. 5,342,774.

In U.S. patent application serial number 938,334, now U.S. Patent No. 5,405,940, the disclosure of which is incorporated by reference, nonapeptides are taught which are presented by the HLA-A1 molecule. The reference teaches that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind one HLA molecule, but not others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

In U.S. Patent Application Serial Number 008,446, filed January 22, 1993 and incorporated by reference, the fact that the MAGE-1 expression product is processed to a second TRA is disclosed. This second TRA is presented by HLA-C clone 10 molecules. The disclosure shows that a given TRAP can yield a plurality of TRAs.

In U.S. Patent Application serial no. 994,928, filed December 22, 1992, and incorporated by reference herein, tyrosinase is described as a tumor rejection antigen precursor. This reference discloses that a molecule which is produced by some normal cells (e.g., melanocytes), is processed in tumor cells to yield a tumor rejection antigen that is presented by HLA-A2 molecules.

In U.S. patent application no. 08/032,978, filed March 18, 1993, and incorporated herein by reference in its entirety, a second TRA, not derived from tyrosinase is taught to be presented by HLA-A2 molecules. The TRA is derived from a TRAP, but is coded for by a non MAGE gene. This disclosure shows that a particular HLA molecule may present TRAs derived from different sources.

In U.S. Patent Application Serial No. 079,110, filed June 17, 1993 and entitled "Isolated Nucleic Acid Molecules Coding For BAGE Tumor Rejection Antigen Precursors" and Serial No. 196,630, filed February 15, 1994, and entitled "Isolated Peptides Which form Complexes with MHC Molecule HLA-C-Clone 10 and Uses Thereof" the entire disclosures of which are incorporated herein by reference, an unrelated tumor rejection antigen precursor, the so-called

"BAGE" precursor, is described. TRAs are derived from the TRAP and also are described. They form complexes with MHC molecule HLA-C-Clone 10.

In U.S. Patent Application Serial No. 096,039, filed July 22, 1993 and entitled "Isolated Nucleic Acid Molecules Coding for GAGE Tumor Rejection Antigen Precursors" and Serial No. 250,162, filed May 27, 1994 and entitled "Method for Diagnosing a Disorder by Determining Expression of GAGE Tumor Rejection Antigen Precursors", the entire disclosures of which are incorporated herein by reference, another unrelated tumor rejection antigen precursor, the so-called "GAGE" precursor, is described. The GAGE precursor is not related to the BAGE or the MAGE family.

In U.S. patent application no. 08/408,015, filed March 21, 1995, and entitled "RAGE Tumor Rejection Antigen Precursors", incorporated herein by reference in its entirety, another TRAP is taught which is not derived from any of the foregoing genes. The TRAP is referred to as RAGE. In U.S. patent application no. 08/530,015, filed September 20, 1995, and entitled "Isolated RAGE-1 Derived Peptides Which Complex with HLA-B7 Molecules and Uses Thereof", also incorporated by reference, the TRA derived form one member of the RAGE family of genes is taught to be presented by HLA-B7 molecules. This disclosure shows that additional TRAPs and TRAs can be derived from different sources.

In U.S. patent application no. 08/253,503, filed June 3, 1994, and entitled "Isolated Nucleic Acid Molecule Which Codes for a Tumor Rejection Antigen Precursor Which is Processed to an Antigen Presented by HLA-B44", incorporated herein by reference in its entirety, another TRAP is taught which is not derived from any of the foregoing genes. The TRAP is referred to as LB33 and later as MUM-1. A tumor rejection antigen is described in the application.

In U.S. patent application no. 08/373,636, filed January 17, 1995, and entitled "Isolated Nucleic Acid Molecule Which Codes for a Tumor Rejection Antigen Precursor Which is Processed to Antigens Presented by HLA Molecules and Uses Thereof", incorporated herein by reference in its entirety, other TRAPs are taught which are derived from LB33/MUM-1 and presented by HLA-B13, HLA-Cw6, HLA-A28 and HLA-A24.

In PCT publication WO96/10577, published April 11, 1996, and entitled "Isolated Nucleic Acid Molecule Coding for a Tumor Rejection Antigen Precursor DAGE and Uses Thereof", incorporated herein by reference in its entirety, another TRAP is taught which is not derived from any of the foregoing genes. The TRAP is referred to as DAGE. A tumor rejection

antigen is described in the application which is presented by HLA-A24.

In U.S. patent application no. 08/487,135, filed June 7, 1995, and entitled "Isolated Nucleic Acid Molecule, Peptides Which Form Complexes with MHC Molecule HLA-A2 and Uses Thereof", incorporated herein by reference in its entirety, another TRAP is taught which is not derived from any of the foregoing genes. The TRAP is referred to as NAG. Various TRAs derived from NAG and presented by HLA-A2 are taught in this application.

In U.S. patent application no. 08/403,388, filed March 14, 1995, and entitled "Isolated Nucleic Acid Molecules Which Are Members of the MAGE-Xp Family and Uses Thereof", incorporated herein by reference in its entirety, three TRAPs are taught which are not derived from any of the foregoing genes. These TRAPs are referred to as MAGE-Xp2, MAGE-Xp3 and MAGE-Xp4.

Other TRAPs include gp100 (Kawakami et al., *J. Immunol.* 154:3961, 1995), gp75 (Wang et al., *J. Exp. Med.* 183:1131, 1996), cdk4 R24C (Wölfel et al., *Science* 269:1281, 1995), and β-catenin (Robbins, *J. Exp. Med.* 183:1185, 1996).

Additional genes similarly are expressed in a tumor specific pattern. Based on the results disclosed in the patents, patent applications and literature described above, it is apparent that a plurality of tumor rejection antigens are derived from any given tumor rejection antigen precursor.

Clinical trials of melanoma antigen-specific immunotherapy have thus far involved the use of either genetically modified tumor cells (Rosenberg et al., *J. Nat'l Cancer Inst.* 86:1159-1166, 1994; Cascinelli et al., *Human Gene Ther.* 5:1059-1064, 1994; Siegler et al., *Human Gene Ther.* 5:761-777, 1994) or the administration of synthetic antigenic peptides known to be presented in conjunction with defined MHC class I molecules (Jager et al., *Int. J. Cancer* 66:162, 1996; Marchand et al., *Int. J. Cancer* 63:883, 1995). Treatments such as administration of synthetic antigenic peptides suffer from the drawback that the HLA type of the subject must be known in order to administer the appropriate tumor rejection antigen peptide. Further, administration of single peptides, or a mixture of peptides, requires extensive characterization of all tumor rejection antigens of a tumor rejection antigen precursor in order to develop treatments for all subjects. This drawback is particularly onerous in view of the continued discovery of additional tumor specific genes which encode tumor rejection antigen precursors.

Thus, in designing treatments effective for a range of subjects having a range of HLA types used in presentation of tumor rejection antigens, methods which are capable of enhancing

the immune response in subjects expressing varied HLA antigen presenting molecules are preferred. An alternative approach to administration of synthetic peptides or genetically modified cells is the use of recombinant viral vectors to deliver the target antigens of choice. In this respect, human adenoviruses have been shown to function effectively as gene transfer vectors in a wide range of cells and tissues. The use of a complete-protein recombinant viral vaccine thus may be preferable because the host alleles can select the relevant epitope or epitopes for presentation. Host epitope selection recently was demonstrated by Zhai et al (*J. Immunol.* 156: 700-710, 1996), wherein tumor infiltrating lymphocytes recognized five different epitopes presented on the surface of cells transduced with a viral vector expressing a cancer antigen.

It is an object of the invention to provide compositions and methods effective in enhancing the immune response of a mammalian subject against tumor cells expressing tumor rejection antigens, in subjects having any type of HLA antigen-presenting molecules. It is a further object of the invention to provide compositions which facilitate the investigation of processing and presentation of tumor rejection antigens.

The invention is elaborated upon further in the disclosure which follows.

Summary of the Invention

The invention provides compositions and methods for using replication-defective adenoviruses or adenovirus genomes to introduce exogenous genetic information (e.g., a nucleic acid encoding a tumor rejection antigen precursor) into a cell via receptor-mediated delivery. According to one aspect of the invention, the adenoviruses or adenovirus genomes are used to deliver a nucleic acid encoding a tumor rejection antigen precursor into a mammalian cell for immunotherapy. In yet another aspect of the invention, the adenoviruses or adenovirus genomes are used to deliver a nucleic acid into a mammalian cell for the production, *in vitro* or *in vivo,* of a nucleic acid transcription or translation product. According to yet other aspects of the invention, compositions containing the adenoviruses or adenovirus genomes of the invention also are provided.

According to one aspect of the invention, an isolated nucleic acid molecule comprising a replication-defective adenovirus genome containing an insert encoding a tumor rejection antigen precursor is provided. In preferred embodiments, the adenovirus genome is a genome of a non-pathogenic adenovirus. Most preferably, the adenovirus genome is an adenovirus type 2 genome or an adenovirus type 5 genome.

In certain embodiments, the adenovirus genome contains at least one inactivated region. The inactivated region is selected from the group consisting of E1, E3 and E4. Preferably, the inactivated region is inactivated by deletion of a portion of the region sufficient to inactivate the at least one region. In preferred embodiments, the at least one inactivated region is E1 and E3. In a particularly preferred embodiment, the adenovirus genome is adenovirus type 5 deletion mutant dl309.

In certain embodiments, the insert encoding a tumor rejection antigen precursor consists essentially of a molecule selected from the group consisting of a nucleic acid molecule encoding MAGE-1, a nucleic acid molecule encoding MAGE-2, a nucleic acid molecule encoding MAGE-3, a nucleic acid molecule encoding MAGE-4, a nucleic acid molecule encoding MAGE-5, a nucleic acid molecule encoding MAGE-6, a nucleic acid molecule encoding MAGE-7, a nucleic acid molecule encoding MAGE-8, a nucleic acid molecule encoding MAGE-9, a nucleic acid molecule encoding MAGE-10, a nucleic acid molecule encoding MAGE-11, a nucleic acid molecule encoding GAGE-1, a nucleic acid molecule encoding GAGE-2, a nucleic acid molecule encoding GAGE-3, a nucleic acid molecule encoding GAGE-4, a nucleic acid molecule encoding GAGE-5, a nucleic acid molecule encoding GAGE-6, a nucleic acid molecule encoding BAGE-1, a nucleic acid molecule encoding RAGE-1, a nucleic acid molecule encoding LB33/MUM-1, a nucleic acid molecule encoding DAGE, a nucleic acid molecule encoding NAG, a nucleic acid molecule encoding MAGE-Xp2, a nucleic acid molecule encoding MAGE-Xp3, a nucleic acid molecule encoding MAGE-Xp4, a nucleic acid molecule encoding tyrosinase, a nucleic acid molecule encoding brain glycogen phosphorylase and a nucleic acid molecule encoding Melan-A. Preferably, the insert consists essentially of a nucleic acid molecule encoding MAGE-1.

According to other preferred embodiments, the insert consists essentially of a nucleic acid molecule encoding at least two tumor rejection antigens. According to certain preferred embodiments, the insert consists essentially of a nucleic acid molecule encoding a tumor rejection antigen precursor capable of being processed into at least two tumor rejection antigens.

In certain embodiments, the isolated nucleic acid molecule includes a non-adenovirus promoter operably linked to the insert encoding the tumor rejection antigen precursor. Preferably, the promoter is non-specific with respect to tissue expression. Preferably, the non-specific promoter is selected from the group consisting of a cytomegalovirus promoter, an adenovirus E1A promoter, an adenovirus MLP promoter, a Rous sarcoma virus LTR promoter,

and a SRα promoter. Most preferably, the promoter is a cytomegalovirus promoter.

In other preferred embodiments, the promoter is selected from the group consisting of a tissue-specific promoter, and a regulatable promoter.

According to another aspect of the invention, a replication-defective adenovirus is provided. The adenovirus comprises a recombinant adenovirus genome, consisting of a nucleic acid molecule as described in any of claims 1-15, enclosed in an adenovirus coat. Preferably, the adenovirus further includes a targeting ligand bound to the adenovirus coat.

According to yet another aspect of the invention, a pharmaceutical preparation comprising an isolated nucleic acid molecule as described in any of the claims 1-15, and a pharmaceutically acceptable carrier is provided.

According to still another aspect of the invention, a pharmaceutical preparation comprising a replication-defective recombinant adenovirus as described in claim 16 or 17 and a pharmaceutically acceptable carrier is provided.

According to a further aspect of the invention, a method for increasing tumor-specific cytolytic T lymphocytes in a subject in need of such treatment is provided. The method includes administering to the subject a replication-defective recombinant adenovirus as described in the claim 16 or 17, wherein the adenovirus genome encodes a tumor rejection antigen precursor. The replication defective recombinant adenovirus is administered in an amount effective to increase cytolytic T lymphocytes specific for the tumor rejection antigen precursor or a tumor rejection antigen derived therefrom. In preferred embodiments, the tumor rejection antigen precursor encoded by the adenovirus genome is selected from the group consisting of MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, MAGE-7, MAGE-8, MAGE-9, MAGE-10, MAGE-11, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, BAGE-1, RAGE-1, LB33/MUM-1, DAGE, NAG, MAGE-Xp2, MAGE-Xp3, MAGE-Xp4, tyrosinase, brain glycogen phosphorylase and Melan-A. Most preferably, the tumor rejection antigen precursor is MAGE-1.

According to still another aspect of the invention, a method for treating a subject with a disorder characterized by expression of a tumor rejection antigen precursor is provided. The method includes administering to the subject an amount of a replication-defective adenovirus encoding the tumor rejection antigen precursor sufficient to ameliorate the disorder. The expression of the tumor rejection antigen precursor by the adenovirus increases cytolytic T lymphocytes specific for complexes of an HLA molecule and a tumor rejection antigen that is

derived from the tumor rejection antigen precursor sufficient to ameliorate the disorder. Preferably, the method further includes administering to the subject an adjuvant.

According to another further aspect of the invention, a method for increasing tumor-specific cytolytic T lymphocytes in a subject in need of such treatment is provided. The method includes administering to the subject a replication-defective recombinant adenovirus genome as described in any of claims 1-15, wherein the adenovirus genome encodes a tumor rejection antigen precursor. The adenovirus genome is administered in an amount effective to increase cytolytic T lymphocytes specific for the tumor rejection antigen precursor or a tumor rejection antigen derived therefrom. Preferably, the method for increasing tumor-specific cytolytic T lymphocytes is performed *ex vivo*. In such a method, a cell of the subject is contacted with the replication-defective recombinant adenovirus genome *ex vivo* and the cell is then administered to the subject.

According to another aspect of the invention, the use of a replication-defective adenovirus or an isolated nucleic acid molecule comprising a replication-defective adenovirus genome containing an insert encoding a tumor rejection antigen precursor in the manufacture of a medicament is provided. Preferred adenoviruses and adenovirus genomes are described above. In certain embodiments, the medicament includes an adjuvant.

According to another aspect of the invention, the use of a replication-defective adenovirus or an isolated nucleic acid molecule comprising a replication-defective adenovirus genome containing an insert encoding a tumor rejection antigen precursor in the manufacture of a medicament for increasing tumor-specific cytolytic T lymphocytes is provided.

The invention also embraces functional variants and equivalents of all of the molecules described above.

These and other aspects of the invention will be described in further detail in connection with the detailed description of the invention.

Brief Description of the Figures

Fig. 1 depicts the construction of the recombinant adenoviruses AdCMVβ-gal and AdCMVMAGE-1.

Fig. 2A depicts the results of a time course of infection of NA8-MEL cells with AdCMVMAGE-1.

Fig. 2B depicts the results of a titration of NA8-MEL cells infected with

AdCMVMAGE-1.

Fig. 3 shows the quantitation of β-galactosidase levels produced in different melanoma lines after transduction by AdCMVβ-gal.

Fig. 4A depicts analyses of MAGE-1 mRNA levels in six melanoma lines transduced with AdCMVMAGE-1.

Fig. 4B depicts analyses of MAGE-1 protein levels in six melanoma lines transduced with AdCMVMAGE-1.

Fig. 5 demonstrates recognition of recombinant adenovirus transduced melanoma cell line NA8-MEL by MAGE-1.A1-specific CTL clones.

Fig. 6 demonstrates recognition of a series of HLA-A1+ melanoma cell lines expressing variable levels of endogenous MAGE-1 mRNA by a CTL clone specific for the MAGE-1.A1 antigen.

Fig. 7 depicts an overall comparison of transduction efficiency, MAGE-1mRNA and protein levels, and susceptibility to lysis by specific anti-MAGE-1.A1 CTLs.

Figs. 8A and 8B demonstrate that AdCMVMAGE-1 transduced melanoma cells can efficiently stimulate the expansion of MAGE-1.A1 specific CTL precursors from autologous peripheral blood lymphocytes (PBL).

Detailed Description of the Invention

The instant disclosure provides replication-defective adenovirus genomes for delivering tumor-specific genes encoding tumor rejection antigen precursors into a target cell, preferably a mammalian cell. An "adenovirus", for the purposes of this invention, refers to an adenovirus that: (1) contains exogenous genetic material that can be transcribed and, optionally, translated in a mammalian cell and (2) contains on its surface a ligand that selectively binds to a receptor on the surface of a target cell, such as a mammalian cell. As used herein, "exogenous genetic material" refers to a nucleic acid molecule (e.g., nucleic acid or oligonucleotide), either natural or synthetic, that is not naturally found in an adenovirus. Preferably, the "exogenous genetic material" is a tumor-specific gene that encodes a tumor rejection antigen precursor that can be processed into a plurality of tumor rejection antigens. Thus, for example, the instant invention embraces the introduction into a mammalian cell of an expression cassette including a recombinant gene containing an inducible promoter operably coupled to a coding sequence of a tumor-specific gene. In the preferred embodiments, the exogenous genetic material of the

adenovirus can be both transcribed and translated in the mammalian target cell.

The genome of an adenovirus is composed of a linear double stranded DNA approximately 36 kilobases in size. The genome comprises, in particular, an inverted repeat sequence (ITR) at each end, an encapsidation sequence (Psi), as well as early genes and late genes. The main early genes are contained in the E1, E2, E3, and E4 regions. Among these early genes, those contained in the E1 region are needed for viral propagation, i.e., replication. The main late genes are contained in the L1 - L5 regions.

The complete nucleotide sequences of adenovirus genomes are know and have been deposited in nucleotide sequence databases. For example, the genome of the adenovirus type 5 has been completely sequenced and is accessible via Genbank accession number M73260. Similarly, portions or even whole genomes of other adenovirus types (type 2, type 7, type 12, and the like) have also been sequenced and deposited in databases.

The nucleic acid encoding a tumor rejection antigen precursor preferably is inserted into a region of the adenovirus genome which is not essential to the production of replication-defective recombinant adenoviruses. For example, the nucleic acid preferably is not inserted into regions which contain adenovirus genes encoding proteins which are not easily supplied *in trans*. Thus, the nucleic acid preferably is inserted into the E1 region, which can be complemented (supplied *in trans*) by a adenovirus encapsidation cell line such as 293 cells. Other preferred sites of insertion of the nucleic acid include the E3 region, which is not required for production of replication-defective recombinant adenoviruses, and the E4 region, mutation of which can be complemented by co-transduction with a helper virus or plasmid or by infection of a suitable complementary cell line. Other sites also may be used as will be apparent to one of ordinary skill in the art. In particular, access to the nucleotide sequences of adenovirus genomes enables a person skilled in the art to identify regions of the adenovirus genome suitable for insertion of the nucleic acid encoding a tumor injection antigen precursor.

The replication-defective recombinant adenoviruses of the invention can be prepared by any technique known to the skilled artisan (Levrero et al., *Gene* 101:195 (1991), EP 185 573; Graham, *EMBO J.* 3:2917 (1984)). Generally, adenoviruses are produced by transfection of a recombinant adenovirus genome into an encapsidation cell line. Where several nucleic acids supply different portions of a replication-defective recombinant adenovirus genome, the several nucleic acids can be cotransduced into the encapsidation cell line. In such cases, the process of forming a replication-defective adenovirus genome involves one or more steps of homologous

recombination between the different nucleic acids transduced into the encapsidation cell line, in order to generate the recombinant adenovirus genome. Generation of an adenovirus by homologous recombination is exemplified in the example (AdCMVβ-gal). After assembly of a complete adenovirus genome (or introduction by transduction a complete adenovirus genome), the adenovirus is assembled by encapsidation of the adenovirus genome with adenovirus coat proteins.

The nucleic acids assembled to prepare a complete replication-defective adenovirus genome can be prepared by any method known in the art. For example, an adenovirus genome can be isolated and then modified *in vitro* by standard methods of molecular biology (see, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York). The modified adenovirus genome so obtained optionally can be isolated and used to transfect an encapsidation cell line. Another technique is based on the use of a plasmid carrying a portion of the genome of the recombinant adenovirus, which plasmid is co-transduced with an adenovirus supplying the missing portions of the genome. A complete recombinant adenovirus genome is formed by homologous recombination in the transduced cell line. Other possibilities include the use of prokaryotic plasmids to prepare the recombinant adenovirus genome, followed by transduction of the plasmids into an adenovirus encapsidation cell line.

The adenovirus encapsidation cell line useful for preparation of recombinant adenoviruses preferably is capable of accepting the nucleic acids described above and preferably contains sequences capable of complementing the replication-defective adenovirus genome to permit production of recombinant adenoviruses. Preferably, such sequences capable of complementing the defective portions of the adenovirus genome are integrated into the genome of the cell to avoid recombination with the adenovirus genome. For example, the human embryonic kidney cell line 293 (Graham et al. *J. Gen. Virol.* 36:59, 1977) contains integrated in its genome the left-hand portion of the genome of a type 5 adenovirus. Other cell lines capable of complementing E1 and E4 functions are described in published PCT applications Nos. WO 94/26914 and WO 95/02697.

Preparation of transduced adenovirus producer cell lines and isolation of adenovirus stock can be performed according to methods standard in the art, as illustrated in the example below.

Exemplary adenoviruses are replication-defective due to mutation of an adenoviral gene

or genes essential for adenoviral replication. As used herein, a "replication-defective" adenovirus is one which is incapable of replicating autonomously in the target cell. Generally, the genome of a replication-defective adenovirus used in the context of the present invention contains mutations or deletions of at least the sequences needed for replication of the adenovirus in the infected cell. Such sequences are well known of those of ordinary skill in the art, and include portions of the E1, E3, and E4 regions of the adenovirus genome. Such regions can be removed in whole or in part, rendered non-functional by mutation, or replaced by other nucleic acid sequences, in particular, a nucleic acid encoding a tumor-rejection antigen precursor. Preferably, the replication-defective adenovirus retains the portions of its genome which are required for encapsidation of the adenovirus genome to form an adenovirus particle. Preferably, the replication-defective adenoviruses of the invention include the inverted repeat sequences (ITRs), a sequence permitting encapsidation, and the nucleic acid encoding a tumor rejection antigen precursor. Replication-defective adenoviruses can contain a modified E1 region which renders such region non-functional. Preferably, the E1 region is deleted in whole or in part, particularly the portion containing open reading frame 3 (ORF3) and open reading frame 6 (ORF6).

Preferably the adenovirus genome used in the invention is derived from a serotype which does not exert pathogenic effects in humans. Preferred adenovirus serotypes for use in the invention include adenovirus type 2 (Ad2) and adenovirus type 5 (Ad5). Other serotypes useful in this manner will be known to persons of skill in the art. Useful adenoviruses (e.g. non-pathogenic adenoviruses) can be prepared by modification of the genome of a pathogenic adenovirus by art-standard recombinant DNA techniques (see, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York). Other adenoviruses useful in the invention include those of non-human origin (see, e.g., PCT application WO94/26914)

The adenoviruses are useful for delivering a nucleic acid encoding a tumor rejection antigen precursor, typically a whole protein, into a mammalian target cell for *ex vivo* and *in vivo* immunotherapy, as well as for producing transcription and translation products of the exogenous genetic material inserted in the recombinant adenovirus genome, in culture or *in vivo*.

The adenoviruses of the invention are also useful for delivering to antigen presenting cells nucleic acid molecules that encode tumor rejection antigen precursors which can be

processed by the antigen presenting cells into at least two tumor rejection antigens and presented at the cell surface to enhance the immune system response of the mammalian recipient to a specific tumor rejection antigen. Exemplary peptide antigens that can be expressed to induce or enhance an immune response are derived from MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, MAGE-7, MAGE-8, MAGE-9, MAGE-10, MAGE-11, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, BAGE-1, RAGE-1, LB33/MUM-1, DAGE, NAG, MAGE-Xp2, MAGE-Xp3, MAGE-Xp4, tyrosinase, brain glycogen phosphorylase and Melan-A.

Optionally, auxiliary nucleic acids are inserted into the adenovirus genome to enhance or otherwise improve the therapeutic efficacy of the immunotherapy in treating the condition. Exemplary auxiliary nucleic acids for delivery to the mammalian target cell include nucleic acids encoding tumor suppressor genes, nucleic acids encoding antisense mRNA or encoding catalytic RNA that inactivate oncogenes, and nucleic acids that render a target tumor cell more susceptible to an administered drug (e.g., suicide genes encoding, for example, thymidine kinase). Auxiliary nucleic acids also include nucleic acids encoding cytokines that enhance a naturally occurring anti-tumor immunity. Exemplary cytokines which have this function include, e.g., IL-4, TNF, IL-2, IL-12 and GM-CSF. Furthermore, nucleic acids encoding costimulatory molecules such as B7-1 and B7-2 can be used (Chamberlain et al., *Cancer Res.* 56:2832-2836, 1996; Townsend and Allison, *Science* 259:368-370, 1993; Chen et al., *J. Exp. Med.* 179:523-532, 1994).

The nucleic acid molecule encoding a tumor rejection antigen precursor is inserted into the adenovirus genome using conventional recombinant DNA techniques, as exemplified in the Example. See also, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. In the preferred embodiments, the nucleic acid molecule encoding a tumor rejection antigen precursor is inserted into well-defined restriction sites in a portion of the adenovirus genome not required for transcription or translation of the nucleic acid molecule. (See, e.g., the Example and figures.) For example, the nucleic acid molecule encoding a tumor rejection antigen precursor preferably is inserted in one of the E1, E3 or E4 regions of adenovirus.

In the preferred embodiments, the adenovirus genome further includes a regulatory sequence, e.g., a promoter region (also referred to as a "promoter"), that is operably coupled to the nucleic acid molecule encoding a tumor rejection antigen precursor. The regulatory sequence controls the expression of the nucleic acid molecule encoding a tumor rejection antigen precursor

in the target cell. As used herein, a nucleic acid molecule encoding a tumor rejection antigen precursor (the TRAP "coding sequence") and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the transcription or the expression of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequence be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequence results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 3' or 5' non-transcribed non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, CAAT sequence, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences can also include enhancer sequences or upstream 5' or downstream 3' transcriptional regulatory sequences as desired.

Exemplary promoters that are useful for protecting the instant invention are provided in Table III, which shows both constitutive promoters and regulatable promoters (e.g., cell lineage specific promoters, inducible promoters). Exemplary constitutive promoters also are included in Table III. In the preferred embodiments, the constitutive promoter is selected from the group consisting of a promoter of the cytomegalovirus, a long terminal repeat (LTR) of retroviruses, e.g., Rous sarcoma virus or Moloney murine leukemia virus, and adenovirus E1A promoter, an adenovirus MLP promoter and a SRα promoter. Exemplary tissue or cell specific transcriptional regulatory sequences are derived from the genes encoding the following proteins: tyrosinase. lipoprotein lipase, albumin, muscle creatine kinase, keratin (K14/K10), globin gene cluster, immunoglobulin heavy chain gene cluster, and involucrin. Several liver-specific promoters, such as the albumin promoter/enhancer, also have been described (see, e.g., PCT application number

PCT/US95/11456, having international publication number WO96/09074, entitled "Use of a Non-mammalian DNA Virus to Express an Exogenous Gene in a Mammalian Cell," hereinafter W0 96/09074, and the references cited therein). In particular, the alpha-feto protein promoter, can be used to effect expression of a therapeutic polynucleotide(s) in liver tumor cells (but not normal liver cells) for treating liver cancer. Exemplary inducible promoters are identified in Table III and are described in the following references: *Science* 268:1786 (1995); *TIBS* 18:471 (1993); *PNAS* 91:3180 (1994); *PNAS* 90:1657 (1993); *PNAS* 88:698 (1991); *Nature Biotechnol.* 14:486 (1996); and *PNAS* 93:5185 (1996). An exemplary repressible promoter, the tetracycline repressible system, is identified in Table III and is described in *PNAS* 89:5547 (1992).

The adenoviruses optionally contain one or more sequences that are suitable for use in the identification of cells that have or have not been transduced. "Transduction", as used herein, refers to the introduction of the adenovirus genome into the target cell. Markers to identify cells that have been transduced include, for example, genes encoding proteins that increase or decrease resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes having activities that are detectable by standard assays known in the art and genes which detectably (e.g. visibly) affect the phenotype of the transduced target cells, hosts, or plaques. Exemplary genes that are suitable as markers include a *lac*Z gene, a chloramphenicol acetyltransferase gene, an alkaline phosphatase gene, a luciferase gene, and a green fluorescent protein gene.

The replication-defective adenoviruses of the invention can be delivered to a cell of a subject by methods known to those of ordinary skill in the art. For example, when delivering a recombinant adenovirus genome without any associated coat protein, the nucleic acid can be introduced into a cell by transduction using a standard technique such as electroporation, liposome transfection, calcium phosphate precipitation, or a commercially available technology such as the Tfx-50 transfection reagent (Promega Corp., Madison, WI). When delivering a recombinant adenovirus genome in association with an adenovirus coat (i.e. in the form of an infectious adenovirus) to a cell, the cell can be contacted with the adenovirus, and the adenovirus genome can be delivered by receptor-mediated endocytosis via binding of an adenovirus coat protein to a cellular receptor.

Targeting of an adenovirus to a particular cell or tissue can be accomplished by choice of an appropriate ligand/receptor pair which is specific for the cell or tissue, and incorporation of the ligand into the adenovirus coat. Methods for attaching a ligand to a viral coat are disclosed below. Exemplary receptors and ligands are disclosed in Table IV.

In addition to the well-known ligand/receptor pairs for delivering a ligand-labeled component to a particular cell type, novel ligands can be identified using phage display procedures such as those described in Hart, et al., *J. Biol. Chem.* 269:12468 (1994). While such filamentous phages could, of course, never be used to deliver genetic material to a cell (because they are single stranded), this methodology is potentially very useful in the discovery of novel receptor ligand interactions. For example, Hart et al. report a filamentous phage display library for identifying novel peptide ligands for mammalian cell receptors. In general, phage display libraries using, e.g., M13 or fd phage are prepared using conventional procedures such as those described in the foregoing reference. The libraries display inserts containing from 4 to 80 amino acid residues. The inserts optionally represent a completely degenerate or a biased array of peptides. Ligands that bind selectively to a particular type of target cell are obtained by selecting those phages which express on their surface a ligand that binds to the target cell of interest. These phages then are subjected to several cycles of reselection to identify the peptide ligand-expressing phages that have the most useful binding characteristics. Typically, phages that exhibit the best binding characteristics (e.g., highest affinity) are further characterized by nucleic acid analysis to identify the particular amino acid sequences of the peptides expressed on the phage surface and the optimum length of the expressed peptide to achieve optimum binding to the target mammalian cell. Alternatively, such peptide ligands can be selected from combinatorial libraries of peptides containing one or more amino acids. Such libraries can further be synthesized which contain non-peptide synthetic moieties which are less subject to enzymatic degradation compared to their naturally-occurring counterparts. These novel ligands can be attached to the adenovirus surface to deliver the virus to the particular target cell of interest.

At least four general procedures are available for attaching a ligand to the surface of an adenovirus. These include (1) chemical modification of the adenovirus surface (e.g., galactosylation, cross linking reactions); (2) modification of the adenovirus genome to express a ligand on the adenovirus surface (e.g., a fusion protein formed between the ligand and a functional viral packaging protein); (3) selective binding of a ligand (e.g., a monoclonal antibody, a polyclonal antibody, or functionally active fragments thereof containing an Fc domain) to an adenovirus surface antigen to mediate targeting of the adenovirus to cells that express an Fc receptor on their surface; and (4) modification of the adenovirus genome to form an avidin-labeled adenovirus intermediate to which a biotinylated ligand (e.g., antibody) can be

attached.

The adenoviruses of the invention are contacted with the target cell under conditions to permit selective binding of the ligand on the surface of the adenovirus to the receptor on the surface of the target cell and to allow the adenovirus to enter the target cell. Conditions which permit the binding of a receptor to its cognate ligand are the physiological conditions (e.g., the particular pH, ionic strength, viscosity) at which the ligands and receptors are known to bind to one another *in vivo* and the conditions at which the ligands and receptors are known to bind to one another *in vitro*, such as in receptor assays for determining the presence of a ligand in, for example, a biological fluid. Such conditions are known to those of ordinary skill in the art of receptor-mediated processes, such as receptor-based binding assays and receptor-mediated delivery of therapeutic agents to preselected tissues *in situ*.

In general, the conditions that allow the target cell to live and transcribe the nucleic acid molecule encoding a tumor rejection antigen precursor are the same conditions that permit selective binding of the ligand to the receptor and that allow the adenovirus to enter the target cell. Optionally, the conditions that allow the cell to transcribe the nucleic acid molecule further include the addition of an inducer that activates an inducible promoter (see, e.g., Table III) to induce transcription and translation of the nucleic acid molecule.

The optimum conditions for inducing the transcription and translation of a nucleic acid molecule encoding a tumor rejection antigen precursor that is under the control of a particular inducible promoter can be determined by one of ordinary skill in the art using no more than routine experimentation. In general, for *in vitro* gene therapy, conventional tissue culture conditions and methods are used to sustain the mammalian cell in culture. For example, the mammalian cell can be allowed to live on a substrate containing collagen, e.g., type I collagen, or a matrix containing laminin, such as described in PCT application number PCT/US95/11456, having international publication number WO96/09074, entitled "Use of a Non-mammalian DNA Virus to Express an Exogenous Gene in a Mammalian Cell," and the references cited therein.

As used herein, "contacting", in reference to the adenovirus and the target cell, refers to bringing the adenovirus into sufficiently close proximity to the target cell to permit the receptor on the target cell to selectively bind to the ligand on the adenovirus. Such conditions are well known to those of ordinary skill in the art. See also, e.g., U.S. patent No. 5,108,921, issued to Low et al. which reports the conditions for receptor-mediated delivery of "exogenous molecules" such as peptides, proteins and nucleic acids to animal cells and U.S. patent No. 5,166,320, issued

to Wu et al., which reports the conditions for the receptor mediated delivery of a ligand-gene conjugate to a mammalian cell. For a further discussion of the conditions and mechanisms by which receptor mediated delivery can be used to deliver an exogenous molecule into a target cell, and in particular, into a mammalian cell, see, e.g., S. Michael, et al., *J. Biol. Chem.* 268(10):6866 (1993), "Binding-incompetent Adenovirus Facilitates Molecular Conjugate-mediated Gene Transfer by the Receptor-mediated Endocytosis Pathway"; M. Barry, et al., *Nature Medicine* 2(3):299 (1996), "Toward cell-targeting gene therapy vectors: Selection of cell-binding peptides from random peptide-presenting phage libraries"; S. I. Michael, *Gene Ther.* 2:660 (1995), "Addition of a short peptide ligand to the adenovirus fiber protein".

The adenovirus can be contacted with the targeted mammalian cell *in vitro*, for example, for *ex vivo* immunotherapy or production of a recombinant protein in cell culture, or *in vivo* for *in vivo* immunotherapy or *in vivo* production of a nucleic acid transcription or translation product. As used herein, a "mammalian target cell" refers to a mammalian cell (preferably, a human cell) which contains on its surface a receptor for the ligand that is contained on the surface of the adenovirus. Essentially, any mammalian cell can be targeted in accordance with the methods of the invention. The cell may be a primary cell or may be a cell of an established cell line. Exemplary cell types that can be targeted in accordance with the methods of the invention are provided in Table IV. Preferably, the mammalian cell is a melanocyte, hepatocyte (liver cell), a breast epithelial cell, a keratinocyte, a hematopoietic cell, e.g., erythrocyte, leukocyte, monocyte, or a lymphocyte.

Where the adenovirus is contacted with the cell *in vitro*, the target cell subsequently can be introduced into the mammal (e.g., into the portal vein or into the spleen) if desired. Accordingly, expression of the nucleic acid molecule encoding a tumor rejection antigen precursor is accomplished by allowing the cell to live or propagate *in vitro*, *in vivo*, or *in vitro* and *in vivo*, sequentially. Similarly, where the invention is used to express a tumor rejection antigen precursor in more than one cell, a combination of *in vitro* and *in vivo* methods are used to introduce the nucleic acid molecule encoding the tumor rejection antigen precursor into more than one mammalian cell.

In *ex vivo* gene therapy, as for *ex vivo* immunotherapy, the cells are removed from a subject and a nucleic acid is introduced into (i.e., transduced) the cells *in vitro*. Typically, the transduced cells are expanded in culture before being reimplanted into the mammalian recipient. The procedure for performing *ex vivo* gene therapy is outlined in U.S. Patent 5,399,346 and in

exhibits submitted in the file history of that patent, all of which are publicly available documents. In general, *ex vivo* immunotherapy involves the introduction *in vitro* of a functional copy of a gene into a cell(s) of a subject which contains a defective copy of the gene, and returning the genetically engineered cell(s) to the subject to stimulate an immune response. The functional copy of the gene is under the operable control of regulatory elements which permit expression of the gene in the genetically engineered cell(s). In *in vivo* immunotherapy, the target cells are not removed from the patient. Rather, the nucleic acid molecule encoding a tumor rejection antigen precursor is introduced into the cells of the mammalian recipient *in situ*, i.e., within the recipient. In general, the methods disclosed herein are practiced by using the replication-defective adenovirus of the invention in place of the gene therapy vectors disclosed in the prior art (e.g., adenoviral vectors, modified adenoviral vectors, retroviral vectors, plasmids, liposomes) in the procedures currently used for administering these vectors (or cells containing these vectors) to the subjects. Such procedures are known to those of skill in the art of human gene therapy.

For *in vivo* immunotherapy, the adenovirus or adenovirus genome is administered to the mammalian recipient, for example, intravascularly, intraluminally (introduction of the adenovirus into body cavities and lumens, such as the genital-urinary tract, gastrointestinal tract, trachea-bronchopulmonary tree or other internal tubular structures), direct injection into a tissue (e.g., muscle, liver), topical application (e.g., eye drops or aerosol application to mucosal surfaces), or intracavitarily (e.g., intraperitoneally or intrathecally (introduction into the cerebrospinal fluid)). Although the ligand/receptor-mediated delivery of the adenovirus is the predominant mechanism for targeting delivery of the adenovirus to a particular cell type, delivery to the target cell can further be modulated by regulating the amount of adenovirus administered to the mammalian recipient and/or by controlling the method of delivery. Thus, for example, intravascular administration of the adenovirus to the portal vein or to the hepatic artery can be used to facilitate targeting the adenovirus to a liver cell.

In general, the adenovirus can be administered to the mammalian recipient using the same modes of administration that currently are used for adenovirus-mediated gene therapy in humans. Such conditions are adequate for contacting the adenovirus and the target cell under conditions to permit selective binding of a ligand on the surface of the adenovirus to a receptor on the surface of the target cell and to allow the adenovirus to enter the target cell. These conditions are described in the following references: *PNAS* 90:10613 (1993); *Nature Medicine* 1:1148 (1995); *Nature Medicine* 12:266 (1996); *New Engl. J. Med.* 333:832 (1995); and *New*

Engl. J. Med. 333:823 (1995). Preferably, the adenovirus is administered to the mammalian recipient by intra-vascular injection, intra-organ introduction by, for example, injection into the organ or contacting the adenovirus with the organ in the presence of a tissue permeabilizing agent; and introduction of the adenovirus into body cavities or lumens. Optionally, immunosuppressive drugs, such as glucocortico-steroids are co-administered with the adenovirus to suppress a primary immune response that may be triggered by an initial exposure to a foreign antigen. Mammalian cells which have been transduced with the adenovirus *ex vivo* can be introduced into the mammalian recipient using the known methods for implanting transduced cells into a human for gene therapy. See, e.g., U.S. Patent No. 5,399,346 ("Gene Therapy") issued to Anderson et al.; PCT International application no. PCT/US92/01890 (Publication No. WO 92/15676, "Somatic Cell Gene Therapy", claiming priority to U.S. Serial No. 667,169, filed March 8, 1991, inventor I. M. Verma); PCT International application no. PCT/US89/05575 (Publication No. WO 90/06997, "Genetically Engineered Endothelial Cells and Use Thereof", claiming priority to U.S. Serial No. 283,586, filed December 8, 1989, inventors Anderson, W.F. et al.).

The invention is not limited in utility to human immunotherapy, but also can be used in the manufacture of a wide variety of proteins and nucleic acids that are useful in the fields of biology and medicine. Moreover, the invention provides a simple method for assessing the processing of tumor rejection antigen precursors, including proper post-translational modifications, *in vitro*. Thus, the invention provides an improved method for introducing a tumor rejection antigen precursor into a mammalian cell. The improved method involves contacting the adenoviruses of the invention (which contain the exogenous nucleic acid molecule encoding a tumor rejection antigen precursor) with the mammalian cell and allowing the adenovirus to enter the cell and transcribe and translate the nucleic acid therein. As discussed above, the adenovirus contains on its surface a ligand that selectively binds to a receptor for the ligand that is contained on the surface of the mammalian cell. The adenovirus genomes of the invention, without the adenovirus coat, also can be used for such methods.

The invention provides other compositions and kits which are useful for practicing the above-described methods. According to a particularly preferred aspect of the invention, an adenovirus of the invention is provided. The adenovirus contains (a) an adenovirus genome containing a nucleic acid molecule insert that encodes a tumor rejection antigen precursor that can be transcribed in a mammalian cell; and (b) an adenovirus coat including a ligand contained

on the surface of the adenovirus coat that selectively binds to a receptor expressed on the surface of a mammalian cell. The adenoviruses of the invention optionally are contained in a pharmaceutically acceptable carrier to form a pharmaceutical composition.

As part of the immunization protocols, substances which potentiate the immune response may be administered with the adenoviruses or adenovirus genomes of the invention. Such immune response potentiating compounds can be classified as either adjuvants or cytokines. Adjuvants may enhance the immunological response by providing a reservoir of antigen (extracellularly or within macrophages), activating macrophages and stimulating specific sets of lymphocytes. Adjuvants of many kinds are well known in the art; specific examples include MPL (SmithKline Beecham), a congener obtained after purification and acid hydrolysis of *Salmonella minnesota* Re 595 lipopolysaccharide, QS21 (SmithKline Beecham), a pure QA-21 saponin purified from *Quillja saponaria* extract, and various water-in-oil emulsions prepared from biodegradable oils such as squalene and/or tocopherol. Cytokines are also useful in vaccination protocols as a result of lymphocyte stimulatory properties. Many cytokines useful for such purposes will be known to one of ordinary skill in the art, including interleukin-12 (IL-12) which has been shown to enhance the protective effects of vaccines (*Science* 268: 1432-1434, 1995).

The pharmaceutical compositions should be sterile and contain a therapeutically effective amount of the adenoviruses (or target cells containing the adenoviruses) in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

The doses of adenovirus or adenovirus genome adminstered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the tumor rejection antigen precursor to be expressed and the desired period of treatment. In general, the replication-defective adenoviruses of the invention are formulated and administered in the form of doses between 10^4 and 10^{14} plaque forming units (pfu), and preferably 10^6 to 10^{10} pfu. Plaque forming units

correspond to the infectious power of an amount of virus. The pfu value of an adenovirus solution can be determined by infecting a suitable cell culture and measuring, after a time sufficient to allow adenovirus-mediated cell lysis, the number of plaques of infected cells. This and other techniques for determination of the pfu value of an adenovirus stock are know to those of ordinary skill in the art.

EXAMPLE: Construction and Characterization of a Recombinant Adenovirus Directing Expression of the MAGE-1 Tumor Specific Antigen

Materials and Methods

Cell lines and clones

The melanoma cell lines Me 222.6, Me 242.B1, Me 256.M3 and Me 260.LN were all established from metastases in our laboratory. The lines MZ2-MEL.3.0, MZ2-MEL.3.1 and MZ2-MEL.2.2 were gifts from the Ludwig Institute for Cancer Research, Brussels Branch, Belgium, and the line NA8-MEL was provided by F. Jotereau (Nantes, France). The 293 cells were obtained from the European Collection of Cell Cultures (ECACC - Wiltshire, UK). All cells were maintained in RPMI 1640 medium containing 10% FCS.

CTL clones 82/30 and 258/8 were kindly provided by P. Coulie (Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium) and were maintained in culture by weekly stimulation with irradiated autologous MZ2-MEL.43 melanoma cells and irradiated EBV transformed lymphoblastoid cell line ROSI in Iscove's medium (GIBCO BRL, Gaithersburg, MD) supplemented as described (Romero et al., *J. Exp. Med.* 182:1019-1028, 1995)

Construction of recombinant adenoviruses

The AdCMVβ-gal recombinant virus was constructed by ligating a 3.5-kb *Hind*III/*Bam*HI fragment containing the *E. coli LacZ* gene into the viral plasmid pACCMV#95 (derived from pACCMVpLpASR- made by R.D. Gerard (Becker et al., *Meth. Cell Biol.*, 1994)) to produce pACβ-gal. This plasmid contains the 5' 6242-bp of Ad-5 from which the region 454 - 3328-bp has been deleted (the deletion incorporates all of the EIA region and part of the EIB region). The plasmid includes a CMV intermediate-early promoter upstream of the cloning site and an SV40 polyadenylation signal downstream. pACO-gal was co-transfected together with

JMI7 (containing an Ad-5 viral backbone) into 293 cells using calcium phosphate precipitation (Becker et al., *supra*; Graham et al., *Mol. Biotechnol.* 3:207-220, 1995). Homologous recombination between the two plasmids resulted in the production of infectious recombinant adenovirus expressing the bacterial β-galactosidase protein. For construction of the AdCMVMAGE-1 virus, a 1.6-kb *MAGE-1* fragment was removed via *Eco*RI sites from a pcDSRα-*MAGE-1* plasmid (provided by B.Van den Eynde, Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium) and cloned into pGEM7Zf(+) (Promega Corp., Madison., WI, USA) to yield pMAGE-7b. The *MAGE-1* gene was removed with *Bam*HI and *Xba*I and ligated into the viral plasmid pSKAC (provided by Karsten Peppel, Duke University. Durham, NC). This plasmid incorporates an Ad-5 left terminal repeat and packaging signal and contains a short polylinker flanked by a CMV promoter and a bovine growth hormone polyadenylation site with transcription terminator. A 2.8-kb *Pme*I/*Xba*I fragment was excised from this plasmid and ligated to an Ad-5 *Xba*I digested viral backbone. The ligated DNA was transfected into 293 cells using the Tfx-50 transfection reagent (Promega Corp.. Madison, WI, USA).

Transfected 293 cells were harvested and individual plaques isolated and purified as described previously (Becker et al., *supra*). Large scale stocks were produced and concentrated before being titered by plaque and limiting dilution assays (Graham et al., *supra*). Concentrated stocks were typically in the range of 10^8 to 10^{10} plaque forming units per milliliter.

Infection of melanoma cells

Cells were seeded and left to incubate overnight before being infected with virus the following morning. All cells were harvested at 48 hours post infection (p.i.) with the exception of the CTL assays and MHC class I surface expression analyses where a 24-hour infection period was used. Unless otherwise stated, the concentrated virus was added directly to the media at a multiplicity of infection (MOI) of 400.

Evaluation of transduction efficiency by E. coli LacZ *expression*

Cells were seeded in six-well culture plates and allowed to attach overnight before infection with AdCMVβ-gal at different MOIs. At 48 hours p.i. cells were fixed in 0.5% gluteraldehyde/PBS at room temperature and then washed twice with PBS. Staining solution containing 2% X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside), 3.3mM potassium

ferricyanide, 3.3mM potassium ferrocyanide, 1mM $MgCl_2$. 60mM Na_2HPO_4, 40mM NaH_2PO_4 and 150mM NaCl (Smythe et al., Annals Thoracic Surg. 57:1395-1401, 1994) was overlayed and the cells were left to incubate at 37°C for 4 hours after which time the proportion of infected cells was estimated by light microscopy.

Quantitation of β-galactosidase expression

The relative levels of β-galactosidase were estimated using the chlorophenol red β-D-galactopyranoside (CPRG) assay system essentially as described (Eustice et al., *Biotechniques* 11:739-740, 1991). Briefly, $6x10^3$ cells were seeded in a 96-well plate and allowed to attach overnight before being infected with AdCMVβ-gal at various MOIs. Cells were incubated for a further 48 hour period and then lysed in 200 µl lysis buffer. 40 µl of each lysate were added to 200µl of assay mixture containing 80mM sodium phosphate buffer, pH 7.3, 10µM 2-mercaptoethanol, 9mM $MgCl_2$ and 8mM CPRG (Boehringer Mannheirn, Rotkreuz, Switzerland). Samples were left at 37°C for 20 minutes and the absorbance measured at 570nm on a Dynatech MR7000 Elisa Reader (Dynatech, Embrach-Embraport, Switzerland).

RNA isolation and Northern blotting

RNA was extracted from infected cells using the Trizol method (GIBCO/BRL - Life Technologies, Gaithersburg, MD) and 10 µg were electrophoresed on a formaldehyde/1% agarose gel before being transferred to Hybond N+ membrane (Amersham Life Science, Buckinghamshire, UK). Prehybridization was done at 65°C for 30 minutes in 7% SDS, 1mM EDTA. 0.5M $NaPO_4$, pH 7.2, after which time a random-primed DNA probe (Random Primed DNA Labelling Kit, Boehringer Mannheim, Rotkreuz, Switzerland) was added and the solution left to hybridise overnight. A full length *MAGE-1* cDNA and a 450-bp fragment of the rat *GAPDH* gene were used as probes. The nylon filter was washed twice at room temperature in 2X SSC; 0.1% SDS for 15 minutes, and then once at 50°C in 0.2X SSC; 0.1% SDS for 15 minutes before being exposed to film. The relative amounts of RNA loaded in each lane were verified by ethidium bromide staining of the gel or, alternatively, the filters were stripped by immersion in a boiling solution of 0.5% SDS and re-hybridized with a *GAPDH* probe.

Protein extraction and Western blotting

Cells were lysed in Laemmli buffer and approximately 25 µg of protein (assessed by

BCA protein assay; Pierce Chemical Co., Rockford, IL) were analyzed by SDS-PAGE on 9% acrylamide minigels (Mini-Protean II, Bio-Rad Laboratories, Richmond, CA). An electroblotting apparatus was. used (70V, 15mA, 4 hours, 4°C) to transfer the protein to Hybond ECL nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK). The anti-MAGE-1 monoclonal antibody 6CI (Carrel et al., *Int. J. Cancer* 67:417-422, 1996) and a monoclonal anti-β-actin mouse antibody (clone AC-40; Sigma Immunochemicals, Sigma Chemie, Switzerland) were used as primary antibodies, and an antimouse Ig horseradish peroxidase-linked whole antibody (NA931, Amersham Life Science, Buckinghamshire, UK) as the secondary antibody. Membranes were blocked overnight in 5% milk powder/PBS at 4°C on a moving platform. They were rinsed briefly with 0.5% Tween20/PBS before being incubated with the primary antibody for 2 hours at 4°C. The membranes were then washed in 0.5% Tween20/PBS three times for 15 minutes each prior to incubation with the secondary antibody for 2 hours. Three more washes in 0.5% Tween20/PBS for 15 minutes each were done and one final wash in PBS for 10 minutes. Proteins were detected using a chemiluminescent system (ECL, Amersham Life Science, Buckinghamshire, UK).

Synthetic peptides and CTL *assays*

Peptide MAGE-1 161-169 was synthesized on solid phase using the standard Fmoc procedures (Romero et al., *supra*). Lysis of target melanoma cells was tested by chromium release assay using standard procedures. Targeted cells were ^{51}Cr labeled for one hour at 37°C in the presence of anti-human class I MHC mAb W6/32 (Brodsky et al., *J. Immunol.* 128:129-135, 1982) in the form of hybridoma culture supernatant and washed three times. Labeled target cells (1000 cells in 150 µl) were then incubated in V-bottom microwells in the presence of various numbers of CTL clones (in 100 µl) and chromium release was measured after 4 hours at 37°C.

Microcultures were set up with the thawed PBL from patient MZ2 (30000 cells/well in round bottom microtiter plates) and stimulated with either irradiated MZ2-MEL.3.1 (autologous melanoma line expressing MAGE-1 antigen, 144 microcultures) or irradiated MZ2-MEL.2.2 transduced with AdCMVMAGE-1 (autologous melanoma subline which lost expression of MAGE- 1 antigen, 144 microcultures) (3000 cells/well) in the presence of IL-7 (10ng/ml) and IL-12 (10u/ml). Microcultures were restimulated on day 10 with the same irradiated stimulator cells and IL-2. On day 20, the lytic activities of aliquots of all microcultures were tested on HLA-A1 homozygous EBV.LCL BM21 in the absence or in the presence of 1µM of antigenic

peptide MAGE- 1 161-169. The cut off value above which a microculture was considered as positive was determined as the average of the sum of all 144 lytic activity values obtained on the BM21 target alone plus 3 SD.

Flow cytometry analysis

Melanoma cells were detached with 0.02% EDTA in PBS and incubated with biotinylated specific monoclonal antibodies followed by phycoerythrin-conjugated streptavidin (Catlag Laboratories, San Francisco, CA) as described (Carrel et al., *Tissue Antigens* 43:110-115, 1994). Labeled cells were analyzed using a FACSII instrument (Becton Dickinson, Mountain View, CA). The following mouse monoclonal antibodies were used: B9.12.1, anti HLA-A, B, C, 6B11, anti-HLA-A1 (Carrel et al., *Int. J. Cancer* 67:417-422, 1996), BB7.2, anti-HLA-A2. Background fluorescence was determined by using a non-reactive antibody.

Results

Construction of the AdCMV β-gal and AdCMVMAGE-1 recombinant adenoviruses.

As depicted in Figure 1, AdCMVβ-gal was constructed by homologous recombination and AdCMVMAGE-1 by direct ligation. Expression of the β-galactosidase and MAGE-1 inserts is driven by the CMV intermediate-early promoter which has been shown to be one of the most potent transcriptional activators having little tissue or species specificity (Boshart et al., *Cell* 41:521-530, 1985). Both viruses contain an adenovirus type-5 (Ad-5) dl309 backbone and have deletions of the E1 regulatory region and partial deletions of the E3 region (Jones et al., *Cell* 13:181-188, 1978). The loss of E1 function renders the recombinant viruses replication defective and accordingly they must be propagated in 293 cells which are able to provide these functions *in trans*. Individual clones isolated from the initial virus pool were tested for expression by Northern blotting and a single clone was chosen for each virus and expanded to produce a large scale concentrated stock.

Expression of AdCMVMAGE-1 in NA8-MEL melanoma cells.

To determine the optimal infection time for expression of MAGE-1 protein a time course was performed. NA8-MEL cells which lack expression of any endogenous MAGE-1 protein, were infected at an MOI of 400 and harvested at different time intervals post infection. Northern

and Western analyses were carried out in parallel to determine the levels of RNA and protein expression respectively (Figure 2A.). MAGE-1 mRNA and protein could be detected as early as 12 hours p.i., and levels of both increased up to a peak at 48 hours p.i. which was sustained through to 72 hours p.i. Incubation of NA8-MEL protein extracts from cells that were transduced with AdCMVMAGE-1 with the 6CI anti-MAGE-1 antibody revealed two different sized proteins - one which corresponds to the expected MAGE- 1 protein (45kDa) and another smaller protein which migrates to about the 39kDa position. This additional protein is detectable in all six melanoma lines, but only after transduction with AdCMVMAGE-1 (Figure 4B). It is unclear what this protein represents; however, it is also recognized by a second MAGE-1 specific monoclonal antibody which detects a different epitope located at the NH_2-teminal flank. Hence, this second immunoactive band in the Western blot may correspond to a truncated form of the full length MAGE-1 protein.

It was also important to determine the effect of varying, the MOI for these melanoma cells since the efficiency of adenoviral transduction has been shown previously to vary between similar as well as different cell types (Gomezmanzano et al., *Cancer Res.* 56:694-699, 1996; Smythe et al., *supra*; Bonnekoh et al., *J. Invest Dermatol.* 104:313-317, 1995). NA8-MEL cells were infected at MOIs of 40, 80, 200, 400 and 800 and harvested at 48 hours p.i. (Figure 2B.). There was no significant toxicity at any of these MOI as determined by cell counts and trypan blue staining. In Northem and Westem analyses, the levels of adenoviral-derived MAGE-1 mRNA and protein increased in a dose dependent manner across the range of MOIs tested.

Relative transduction efficiency between different melanoma cell lines

In order to determine the relative transduction efficiencies among different melanoma cell lines, cells were infected with AdCMVβ-gal and then stained for LacZ expression using the β-galactosidase substrate, X-gal (Table I). Surprisingly, there was significant variation in the transduction efficiencies for the eight lines tested despite each having been derived from human melanoma metastases. Over the five MOIs examined, the Me 242.B. 1 and NA8-MEL lines were infected the most efficiently, with the Me 222.6 and MZ2-MEL.2.2 lines being transduced the least efficient. The remaining four lines all exhibited intermediate transduction efficiencies.

To establish the relationship between the efficiency of transduction and the relative level of protein synthesized, cells from six melanoma lines were transduced with AdCMVβ-gal and lysed 48 hours p.i. The amount of β-galactosidase was assayed in the presence of CPRG by

measuring the relative absorbance at 570nm (Figure 3). The levels of β-galactosidase for all cell lines increased in a MOI dependent manner up to the highest MOI tested (800 pfu/cell). These data correlate well with the previous and together indicate that the cells that are transduced most efficiently synthesize the highest levels of β-galactosidase protein.

Comparison of AdCMVMAGE-1 RNA and protein levels in melanoma cell lines

It was important to investigate the relationship between the MAGE-1 RNA and protein levels in the AdCMVMAGE-1 transduced cells. Six cell lines were infected at an MOI of 400 and incubated for 48 hours. RNA was extracted from infected cells as well as uninfected controls and hybridized to a MAGE-1 random-primed probe (Figure 4B.). To standardize the relative levels of RNA, the filter was stripped and reprobed with a *GAPDH* fragment and the relative intensities for each lane were quantitated using a phosphorimager (Applied Biosystems). Additional dishes with the six tumor cell lines were seeded and infected at the same MOI and left for the same length of time. Protein was extracted and transferred to a membrane which was subsequently incubated with the anti-MAGE-1 antibody 6Cl and then stripped and reincubated with a mouse anti-β-actin antibody as an internal control (Figure 4B). The relative levels of MAGE-1 protein were determined by scanning densitometry (Hirschmann Elscript 400). Figure 7 depicts the levels of RNA and protein for each cell line compared to the designated standard, MZ2-MEL.3.0. The levels of adenoviral-derived MAGE-1 mRNA correlate closely with the amounts of MAGE-1 protein for all six cell lines suggesting that there are no major differences in post-transcriptional modifications between the different lines.

Recognition of a series of HLA-A1+ melanoma cell lines expressing variable levels of MAGE-1 mRNA by CTL clones specific for the MAGE-1.A1 antigen.

To examine whether the adenovirus derived MAGE-1 protein could be processed and presented correctly in the context of the HLA-A1 molecule to specific CTL, a cytotoxicity assay was done using two MAGE-1.Al specific cytolytic T lymphocyte (CTL) clones (82/30 and 258/8). Cells were infected (400 MOI) with AdCMVMAGE-1 and harvested for chromium labeling at 24 hours p.i. Both CTL clones were able to lyse about 35% of transduced NA8-MEL cells (Figure 5). The other five melanoma cell lines were also transduced and incubated with the 82/30 clone. The levels of specific lysis ranged from 38 percent for Me 222.6 up to 88 percent for Me 242.B.1. In most cases the susceptibility of the AdCMVMAGE-1 transduced cells for

either CTL clone was comparable to that achieved by pre-pulsing with the synthetic MAGE-1.A1 peptide (EADPTGHSY; SEQ ID NO:1). Infection with AdCMVβ-gal and the addition of a synthetic MAGE-3 peptide which has been previously identified as an anti-MAGE-3 CTL epitope (Gaugler, *J. Exp. Med.* 179:921, 1994) did not give rise to any recognition indicating the lysis was specific to MAGE-1 expressing cells.

Effects of transduction on MHC class I expression

Of the six transcription units identified in the adenovirus E3 region, three have been demonstrated to be able to modulate the host immune response (Wold et al., *Trends Microbiol.* 2:437-443, 1994) whilst the function of the remaining three remains unknown. The gp19K protein is the most abundant E3 product and serves to lower the number of MHC class I cell surface complexes available for antigen presentation (Flomenberg et al., *Mol. Immunol.* 31:1277-1284, 1994; Wold et al., *Virology* 184:1-8, 1991; Burgert et al., *EMBO J.* 6:2019-2026, 1987). Since AdCMVβ-gal and AdCMVMAGE-1 are both derived from the Ad-5 deletion mutant dl309 which has been shown to express gp19K protein (Bett et al., *Virus Res.* 39:75-82, 1995), we decided to test transduced cells for any changes in MHC class I expression which might affect recognition by the two anti-MAGE-1.A1 CTL clones. Cell cultures were transduced with AdCMVMAGE-1 at 400 MOI and FACS analysis using anti-MHC class I (B9.12.1), anti-HLA-A1 (6BII) and anti-HLA-A2 (BB7.2) antibodies was carried out at 24 hours p.i. We were unable to detect any significant decrease in surface MHC class I expression, or expression of the HLA-A1 and -A2 antigens between infected and uninfected cells (Table II).

Expansion of MAGE-1.A1 specific CTL precursors from autologous PBL

In order to gauge the potential with which transduced cells might induce the expansion of specific CTLs, the MAGE-1 antigen loss derivative MZ2-MEL.2.2. was infected with AdCMVMAGE-1 and incubated with the CTL clone 258/8. The MAGE-1+ parent cell line MZ2-MEL.3.1 was also incubated with the CTL clone as a positive control. Chromium labeling of the cells indicated significant levels of lysis for both lines (92 and 34 percent, respectively). Microcultures were then set up from patient MZ2 and stimulated with either irradiated MZ2-MEL.3.1 (autologous melanoma line expressing MAGE-1 antigen) or irradiated MZ2-MEL.2.2 (autologous melanoma subline which lost expression of MAGE-1 antigen) which had been transduced with AdCMVMAGE-1. Cells were restimulated on day 10 with the same cells. On

day 14, the lytic activities of aliquots of all microcultures were tested on HLA-A1 homozygous EBV.LCL BM21 in the absence or in the presence of 1μM of antigenic peptide MAGE-1 161-169 (SEQ ID NO:1). The results show an increase in CTL expansion in 10 of the cultures stimulated with the transduced MZ2-MEL.2.2 cells compared to 26 cultures with the MAGE-I positive MZ2-MEL.3.1 cells. Unfortunately, the limited number of patient PBLs did not permit a control experiment without any stimulator cells.

Each of the references, patents and patent publications disclosed in this document is incorporated in its entirety herein by reference.

While the invention has been described with respect to certain embodiments, it should be appreciated that many modifications and changes may be made by those of ordinary skill in the art without departing from the spirit of the invention. It is intended that such modification, changes and equivalents fall within the scope of the following claims.

The Tables are presented below and are followed by a Sequence Listing and what is claimed:

Table I: *Proportion of melanoma cells infected with AdCMVβ-gal at different MOIs*

	Multiplicity of infection (MOI)				
Cell line	40	80	200	400	800
NA8-MEL	2+	3+	4+	5+	5+
MZ2-MEL.3.0	+/-	+	2+	4+	5+
Me 242.B.1	+	2+	3+	4+	5+
Me 256.M3	+	+	2+	4+	4+
Me 260.LN	+	2+	3+	4+	4+
Me 222.6	+/-	+/-	+	2+	3+
MZ2-MEL.3.1	+/-	+	2+	4+	5+
MZ2-MEL.2.2	-	+/-	+/-	+	3+

Experiments were done in triplicate and LacZ expression was scored as: - (no expression), +/- (<1% of cells), + (1-10% of cells), 2+ (10-25% of cells), 3+ (25-50% of cells), 4+ (50-75% of cells), 5+ (75-100% of cells).

Table II: *Effects of recombinant adenovirus AdCMVMAGE-1 on MHC class I expression*

Cell lines	AdCMVMAGE-1	MHC class I	HLA-A1	HLA-A2
MZ2-MEL.3.0	-	143	7	2
	+	154	9	2
NA8-MEL	-	246	21	67
	+	184	28	62
Me 222.6	-	172	13	13
	+	178	15	12
Me 256.M3	-	93	12	7
	+	111	12	7
Me 260.LN	-	138	10	2
	+	154	15	2
Me 242.B.1	-	305	4	32
	+	407	4	47
MZ2-MEL.3.1	-	38	5	9
	+	77	3	14
MZ2-MEL.2.2	-	18	2	5
	+	35	2	4

Mean channel numbers of fluorescence were determined by FACS analysis at 24 hours p.i., and the data shown is from one representative experiment.

Table III: *Exemplary Promoters and Enhancers*

Constitutive
Phosphoglycerokinase
Long terminal repeat (LTR) of retroviruses, e.g. Moloney murine leukemia virus,
Rous sarcoma virus
Cytomegalovirus promoter
Srα promoter (SV40 early promoter-fused HTLV-I LTR)
HPRT
vimentin
α-actin
tubulin

Hematopoeitic cells
Promoters
c fms (monocytes, trophoblasts)
T-cell receptor
Enhancers
Immunoglobulin heavy chain
Locus control region of the globin gene complex CD2

Hepatocytes
Promoters
Albumin
α-1-antitrypsin
Pyruvate kinase
Phosphoenol pyruvate carboxykinase
Transferrin
Transthyrethrin
α-fetoprotein
α-fibrinogen
β-fibrinogen
Hepatitis B
Enhancers
Hepatitis B
Tyrosine aminotransferase

Cardiac myocytes
Promoter
Myosin light chain-2
β-myosin heavy chain (cardiac and slow twitch skeletal)
α-cardiac myosin heavy chain
Cardiac alpha actin
Enhancer
β-myosin heavy chain

Fibroblasts
Promoter
Collagen alpha-2 (I)
Elastin (fibroblasts and smooth muscle cells)

Neurons
Promoter
Peripheral myelin protein-22

Adipocytes
Promoter
Lipoprotein lipase
Aromatase cytochrome P450 (adipocytes, brain, ovary)

Thyroid
Promoter
Thyroglobulin

Lens epithelium
Promoter
Crystallin

Breast epithelium
Promoter
Milk protein gene

Skeletal muscle
Promoter
Glut-4
Muscle creatine kinase (skeletal and cardiac muscle)
Enhancer
Muscle creatine kinase (skeletal and cardiac muscle)

Urinary bladder
Promoter
UroplakinII

Keratinocyte
Promoter
Keratin 14
Keratin 10
Involucrin

Melanocyte
Promoter
Tyrosinase

Non specific enhancer elements
SV40
CMV
LTR

Inducible or repressible promoter systems
Estrogen-Gal4 inducible system
RU486-Gal4 inducible system
Tetracycline inducible system
IPTG system
Metallothionein
Tetracycline repressible system
Steroid hormone receptor
Retinoic acid receptor

Table IV: *Exemplary Receptors and Preferred Ligands*

Hepatic receptors
hyaluronic acid
collagen
N-terminal propeptide of collagen type III
mannose/N-acetylglucosamine
complement
asialoglycoprotein
tissue plaminogen activator
low density lipoprotein
insulin
ceruloplasmin
enterokinase
carcinoembronic antigen
apamin
galactose/lactose

Growth Factor/Cytokine receptors
hepatocyte growth factor
epidermal growth factor
insulin-like growth factor I, II
interleukin-1α/β
interleukin-2, IL-7, IL-4
γ-interferon
β-interferon
keratinocyte growth factor
TNF-R p55

Hormone receptors
prolactin
thyroglobulin
growth hormone
insulin
glucagon
leutinizing hormone
human choriogonadotrophic hormone

Nerve cell receptors
neurotensin

Antigen presenting cell receptors
immunoglobulin G-Fc receptor

Kidney cells
angiotensin II
vasopressin

Bone marrow receptors
c kit
CD-34

Keratinocyte and skin fibroblast receptors
very low density lipoprotein
low density lipoprotein
integrins that bind to RGD bearing polypeptides
collagen
laminin

Placental receptors
hemopexin
immunoglobulin G-Fc
low density lipoprotein
transferrin
alpha2-macroglobulin
ferritin
insulin
γ-interferon
epidermal growth factor
insulin-like growth factor

Muscle cell receptors
insulin
very low density lipoprotein

Gut epithelium
cobalamin-intrinsic factor
heat stable enterotoxin of E. Coli

Breast epithelium
heregulin
prolactin

Melanocytes
c kit

Miscellaneous
folate
cobalamin (B12)

Preferred ligands
low density lipoprotein (apoprotein B100)
very low density lipoprotein (apoprotein E)
galactose
c kit ligand
transferrin

insulin
heregulin
RGD or RGD-containing polypeptides

5

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT:

(A) NAME: LUDWIG INSTITUTE FOR CANCER RESEARCH

(B) STREET:1345 AVENUE OF THE AMERICAS

(C) CITY: NEW YORK

(D) STATE: NEW YORK

(E) COUNTRY: UNITED STATES OF AMERICA

(F) ZIP: 10105

(ii) TITLE OF THE INVENTION: REPLICATION-DEFECTIVE ADENOVIRUSES FOR CANCER IMMUNOTHERAPY

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: WOLF, GREENFIELD & SACKS, P.C.

(B) STREET: 600 ATLANTIC AVENUE

(C) CITY: BOSTON

(D) STATE: MASSACHUSETTS

(E) COUNTRY: UNITED STATES OF AMERICA

(F) POSTAL CODE: 02110

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: DOS

(D) SOFTWARE: FastSEQ Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/027,891

(B) FILING DATE: 07-OCT-1996

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Van Amsterdam, John R.

(B) REGISTRATION NUMBER: 40,212

(C) REFERENCE/DOCKET NUMBER: L0461/7021WO

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-720-3500

(B) TELEFAX: 617-720-2441

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu Ala Asp Pro Thr Gly His Ser Tyr
1 5

CLAIMS

1. An isolated nucleic acid molecule comprising a replication-defective adenovirus genome containing an insert encoding a tumor rejection antigen precursor.

2. The isolated nucleic acid molecule of claim 1, wherein the adenovirus genome is an adenovirus type 2 genome or an adenovirus type 5 genome.

3. The isolated nucleic acid molecule of claim 2, wherein the adenovirus genome contains at least one inactivated region, wherein the inactivated region is selected from the group consisting of E1, E3 and E4.

4. The isolated nucleic acid molecule of claim 3, wherein the at least one inactivated region is inactivated by deletion of a portion of the region sufficient to inactivate the at least one region.

5. The isolated nucleic acid molecule of claim 4, wherein the at least one inactivated region is E1 and E3.

6. The isolated nucleic acid molecule of claim 5, wherein the adenovirus genome is adenovirus type 5 deletion mutant dl309.

7. The isolated nucleic acid molecule of claim 1, wherein the insert encoding a tumor rejection antigen precursor consists essentially of a molecule selected from the group consisting of a nucleic acid molecule encoding MAGE-1, a nucleic acid molecule encoding MAGE-2, a nucleic acid molecule encoding MAGE-3, a nucleic acid molecule encoding MAGE-4, a nucleic acid molecule encoding MAGE-5, a nucleic acid molecule encoding MAGE-6, a nucleic acid molecule encoding MAGE-7, a nucleic acid molecule encoding MAGE-8, a nucleic acid molecule encoding MAGE-9, a nucleic acid molecule encoding MAGE-10, a nucleic acid molecule encoding MAGE-11, a nucleic acid molecule encoding GAGE-1, a nucleic acid molecule encoding GAGE-2, a nucleic acid molecule encoding GAGE-3, a nucleic acid molecule encoding GAGE-4, a nucleic acid molecule encoding GAGE-5, a nucleic acid molecule encoding GAGE-6, a nucleic acid molecule encoding BAGE-1, a nucleic acid molecule encoding

RAGE-1, a nucleic acid molecule encoding LB33/MUM-1, a nucleic acid molecule encoding DAGE, a nucleic acid molecule encoding NAG, a nucleic acid molecule encoding MAGE-Xp2, a nucleic acid molecule encoding MAGE-Xp3, a nucleic acid molecule encoding MAGE-Xp4, a nucleic acid molecule encoding tyrosinase, a nucleic acid molecule encoding brain glycogen phosphorylase and a nucleic acid molecule encoding Melan-A.

8. The isolated nucleic acid molecule of claim 7, wherein the insert consists essentially of a nucleic acid molecule encoding MAGE-1.

9. The isolated nucleic acid molecule of claim 7, wherein the insert consists essentially of a nucleic acid molecule encoding at least two tumor rejection antigens.

10. The isolated nucleic acid molecule of claim 7, wherein the insert consists essentially of a nucleic acid molecule encoding a tumor rejection antigen precursor capable of being processed into at least two tumor rejection antigens.

11. The isolated nucleic acid molecule of claim 1, further comprising a non-adenovirus promoter operably linked to the insert encoding the tumor rejection antigen precursor.

12. The isolated nucleic acid molecule of claim 11, wherein the promoter is non-specific with respect to tissue expression.

13. The isolated nucleic acid molecule of claim 12, wherein the promoter is selected from the group consisting of a cytomegalovirus promoter, an adenovirus E1A promoter, an adenovirus MLP promoter, a Rous sarcoma virus LTR promoter, and a SRα promoter.

14. The isolated nucleic acid molecule of claim 13, wherein the promoter is a cytomegalovirus promoter.

15. The isolated nucleic acid molecule of claim 11, wherein the promoter is selected from the group consisting of a tissue-specific promoter and a regulatable promoter.

16. A replication-defective adenovirus comprising a recombinant adenovirus genome, which consists of a nucleic acid molecule as described in any of claims 1-15, enclosed in an adenovirus coat.

17. The replication-defective adenovirus of claim 16, further comprising a targeting ligand bound to the adenovirus coat.

18. A pharmaceutical preparation comprising:
an isolated nucleic acid molecule as described in any of claims 1-15, and
a pharmaceutically acceptable carrier.

19. A pharmaceutical preparation comprising:
a replication-defective recombinant adenovirus as described in claim 16 or 17, and
a pharmaceutically acceptable carrier.

20. A method for increasing tumor-specific cytolytic T lymphocytes in a subject in need of such treatment, comprising administering to the subject a replication-defective recombinant adenovirus as described in claim 16 or 17, wherein the adenovirus genome encodes a tumor rejection antigen precursor, in an amount effective to increase cytolytic T lymphocytes specific for the tumor rejection antigen precursor or a tumor rejection antigen derived therefrom.

21. The method of claim 20, wherein the tumor rejection antigen precursor is selected from the group consisting of MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, MAGE-7, MAGE-8, MAGE-9, MAGE-10, MAGE-11, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, BAGE-1, RAGE-1, LB33/MUM-1, DAGE, NAG, MAGE-Xp2, MAGE-Xp3, MAGE-Xp4, tyrosinase, brain glycogen phosphorylase and Melan-A.

22. The method of claim 21, wherein the tumor rejection antigen precursor is MAGE-1.

23. A method for treating a subject with a disorder characterized by expression of a tumor rejection antigen precursor, comprising
administering to the subject an amount of a replication-defective adenovirus encoding the

tumor rejection antigen precursor sufficient to ameliorate the disorder, the expression of the tumor rejection antigen precursor by the adenovirus increasing cytolytic T lymphocytes specific for complexes of an HLA molecule and a tumor rejection antigen that is derived from the tumor rejection antigen precursor sufficient to ameliorate the disorder.

24. The method of claim 23, further comprising administering to the subject an adjuvant.

25. A method for increasing tumor-specific cytolytic T lymphocytes in a subject in need of such treatment, comprising administering to the subject a replication-defective recombinant adenovirus genome as described in any of claims 1-15, wherein the adenovirus genome encodes a tumor rejection antigen precursor, in an amount effective to increase cytolytic T lymphocytes specific for the tumor rejection antigen precursor or a tumor rejection antigen derived therefrom.

26. The method of claim 25, wherein a cell of the subject is contacted with the replication-defective recombinant adenovirus genome *in vitro* and the cell then is administered to the subject.

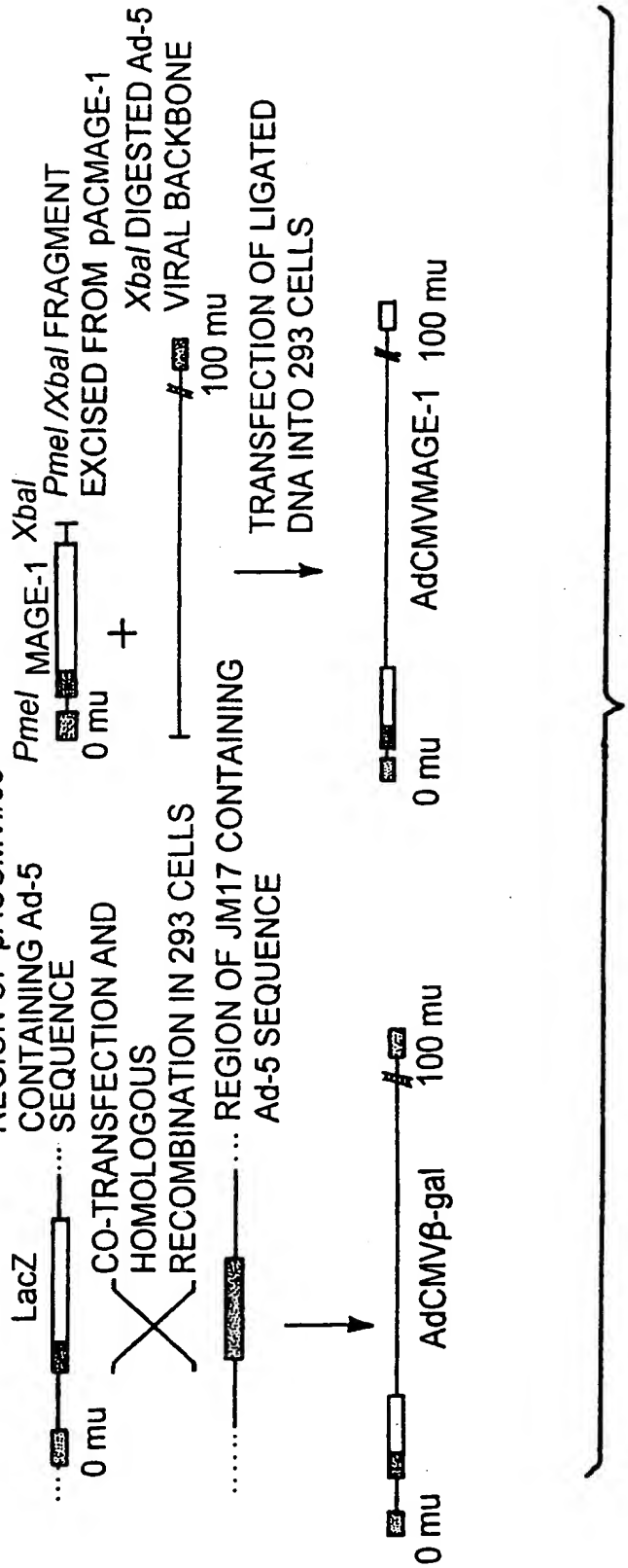


Fig. 1

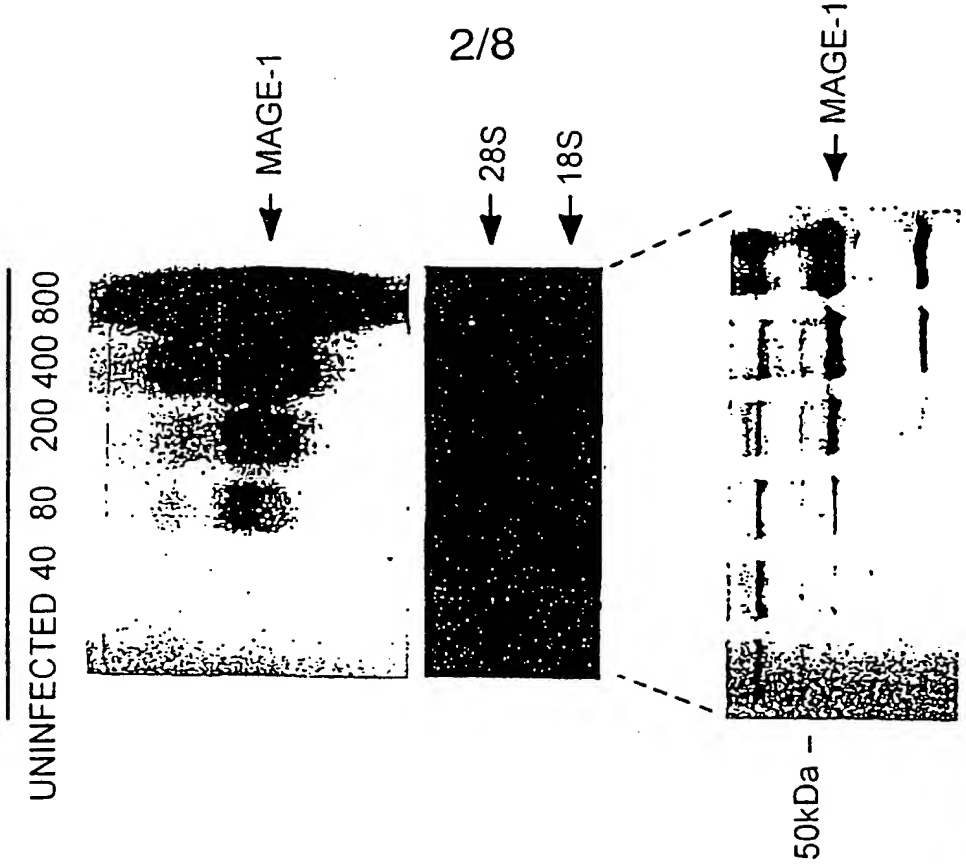


FIG. 2B

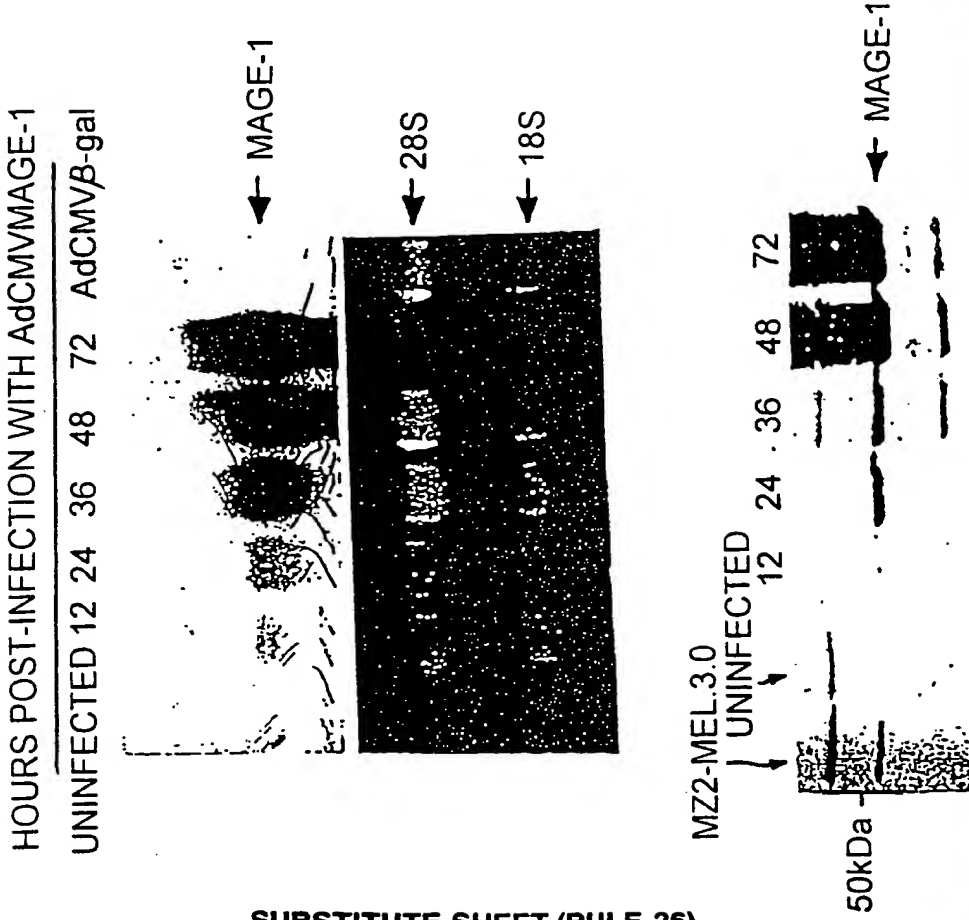


FIG. 2A

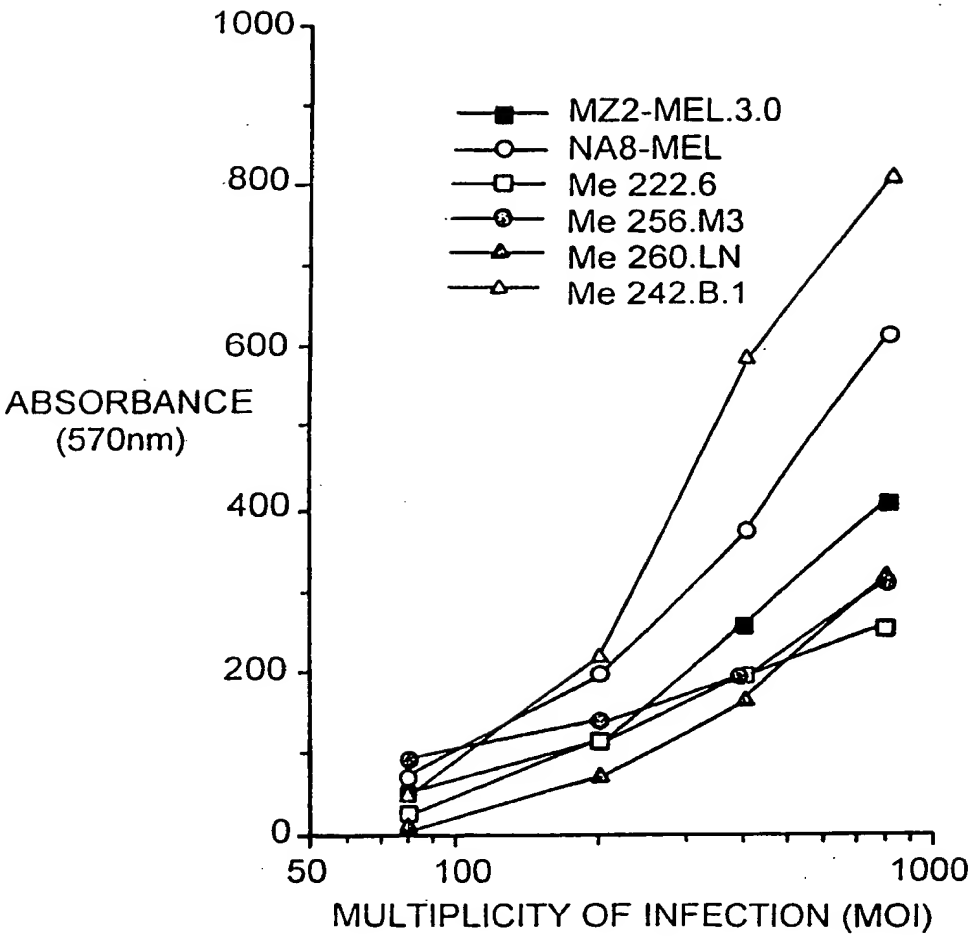


Fig. 3

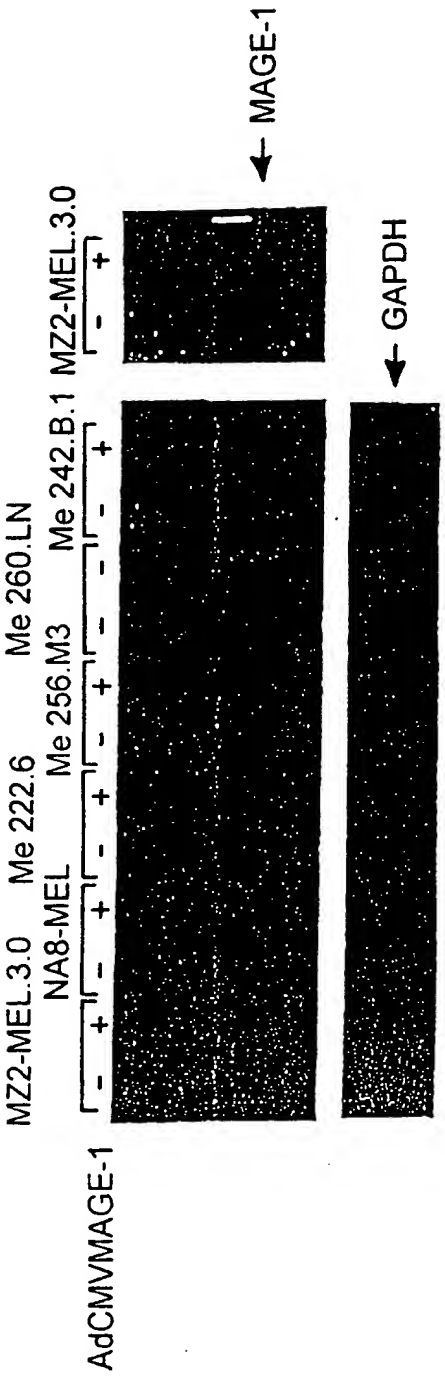


FIG. 4A

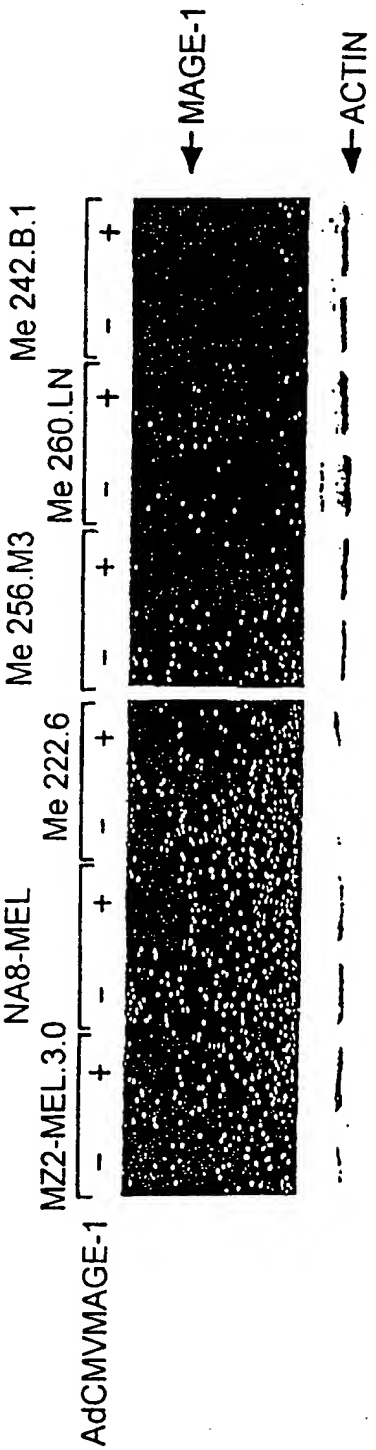


FIG. 4B

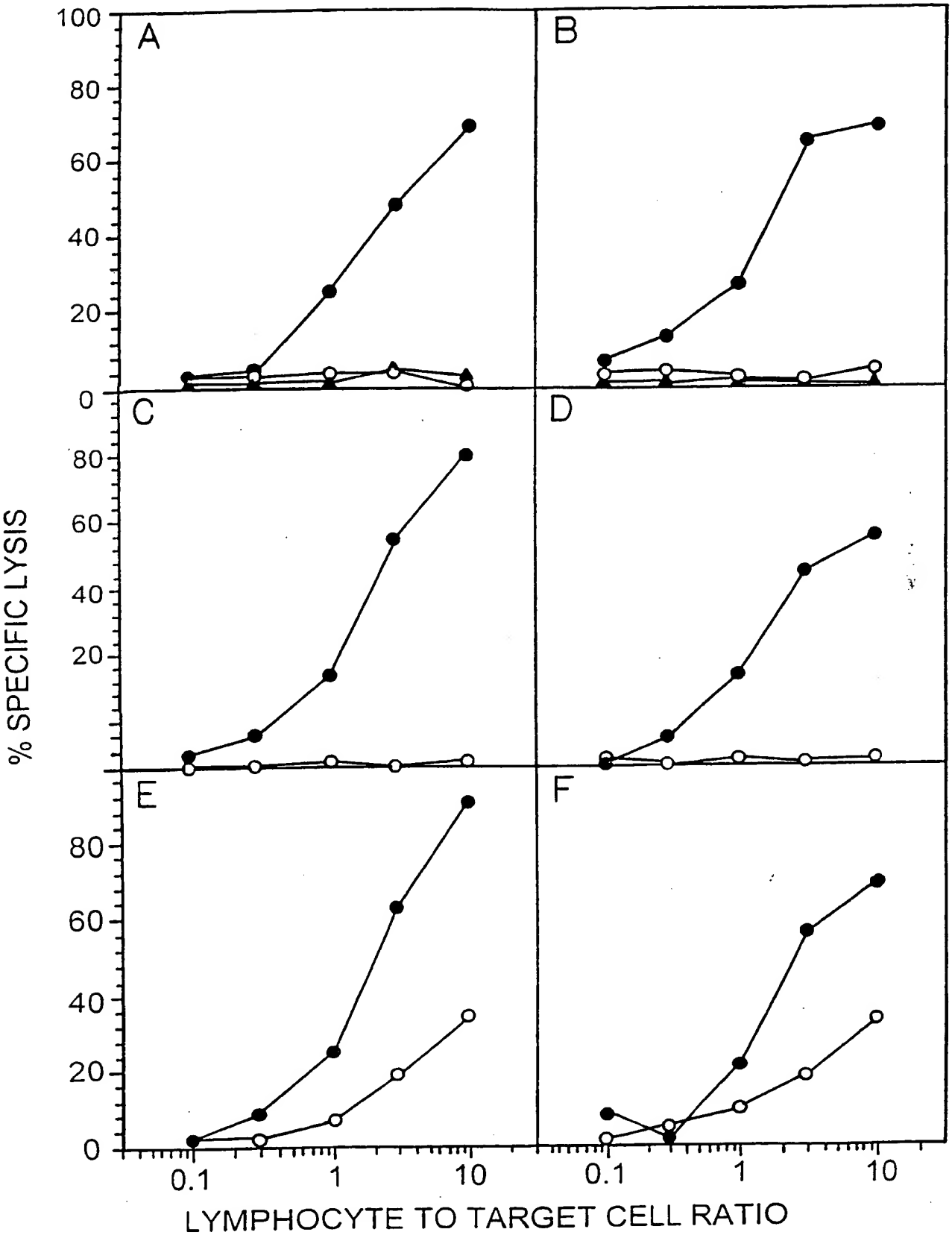


Fig. 5

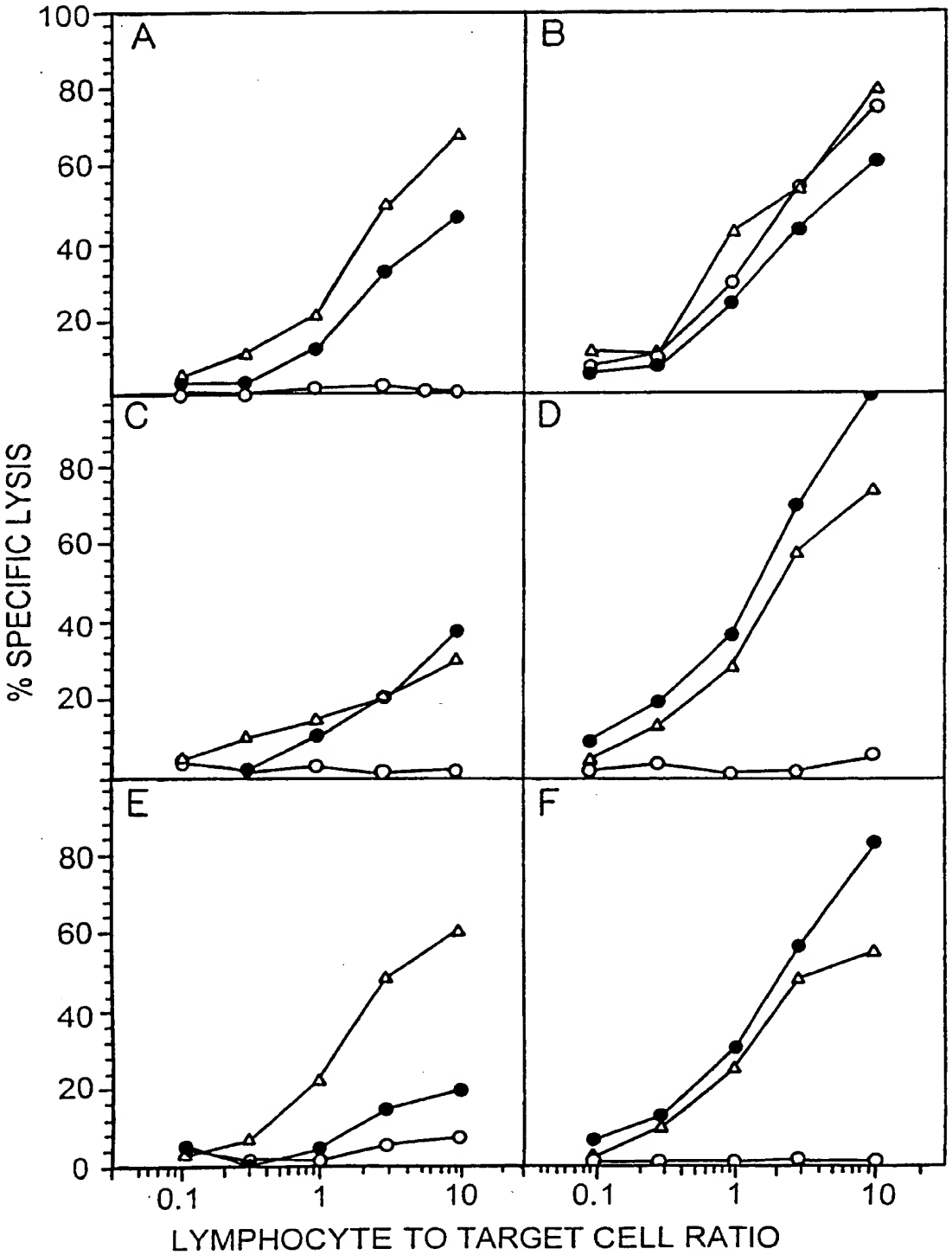


Fig. 6

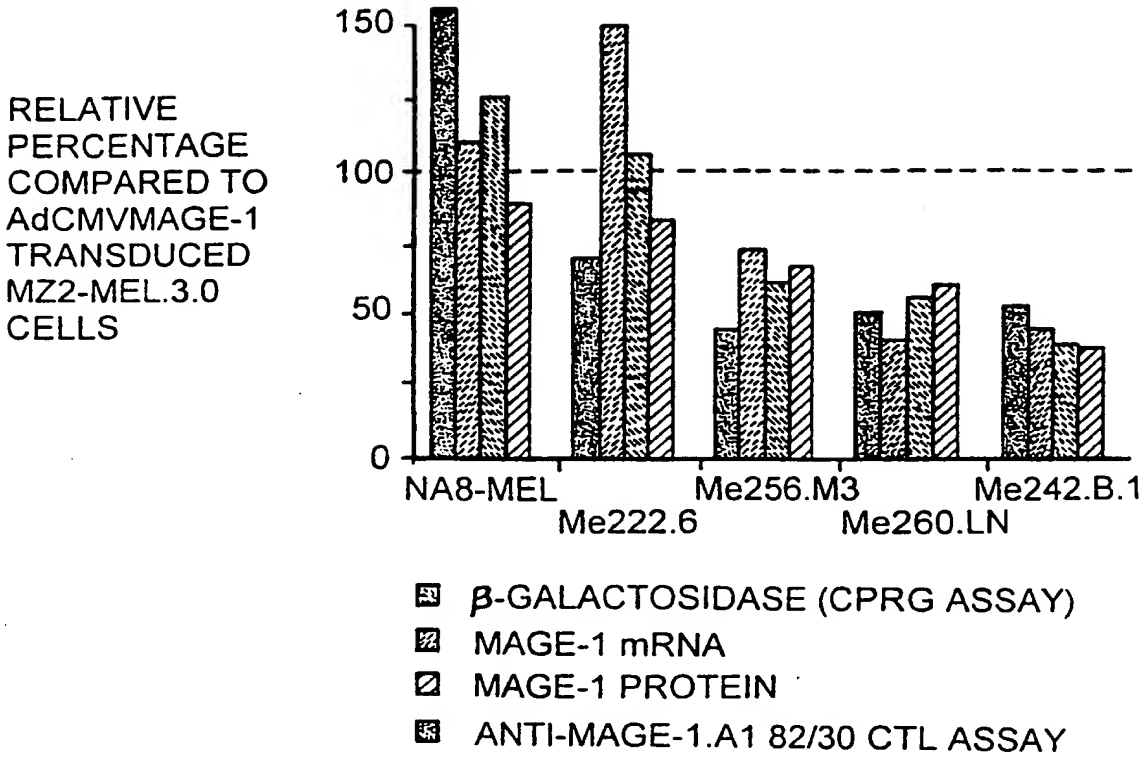


Fig. 7

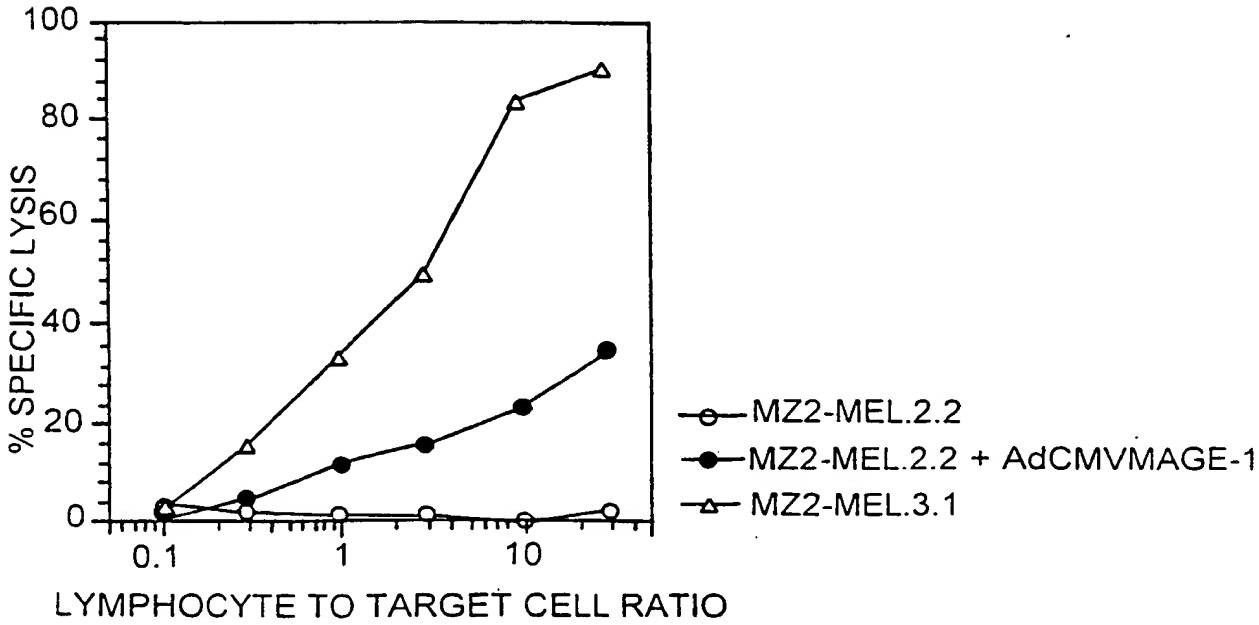


FIG. 8A

STIMULATOR CELL	TARGET CELL (BM 21)	1 1 3 1 1 3 3 4 1 1 0 2 0 1 2 0 2 0 1 1 0 0 1 0 2 2
		11 8 6 14 5 14 36 32 13 7 15 29 10 7 22 14 35 5 22 22 23 7 12 9 26 5
MZ2-MEL.3.1	ALONE	
	+MAGE-1 161-169	
MZ2-MEL.2.2 INFECTED WITH AdCMVMAGE-1	ALONE	0 0 2 1 0 1 1 1 0 0
	+MAGE-1 161-169	8 10 13 6 4 35 8 38 23 10

FIG. 8B

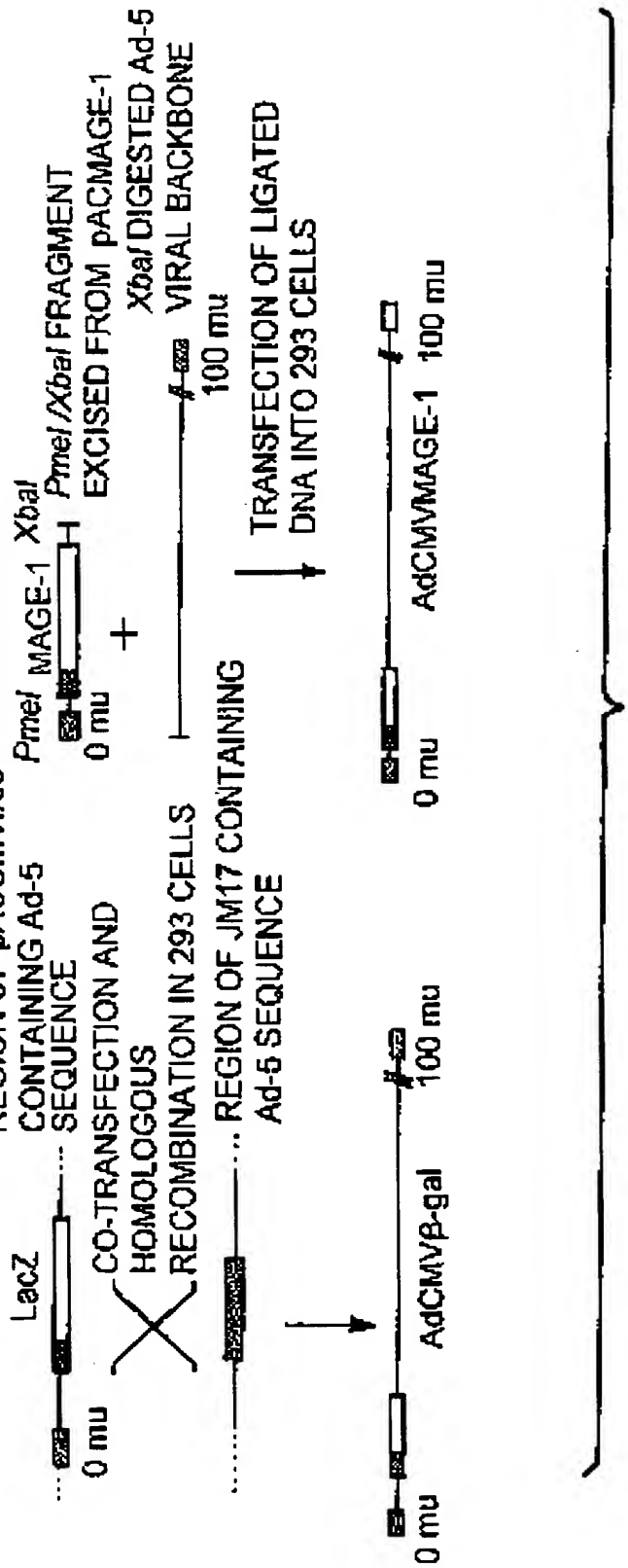


Fig. 1

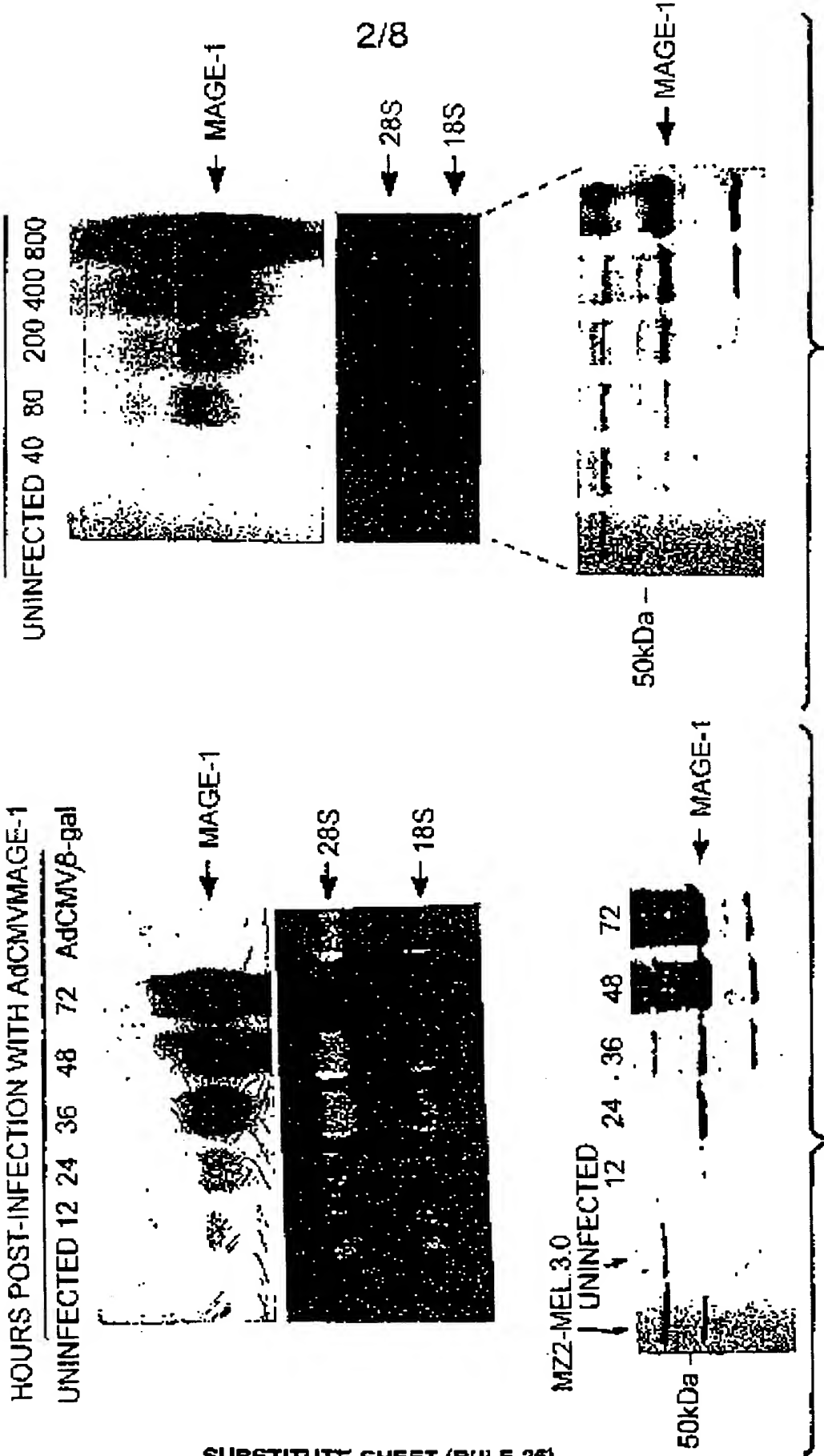


FIG. 2B

FIG. 2A

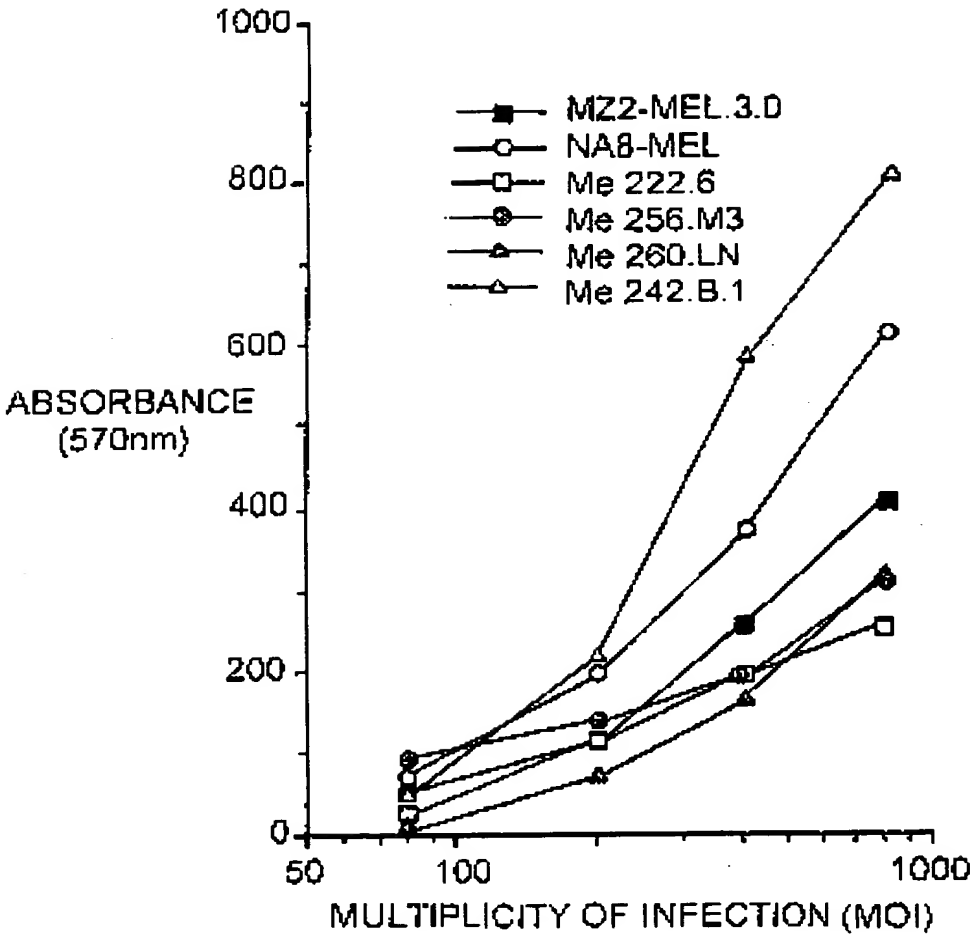


Fig. 3

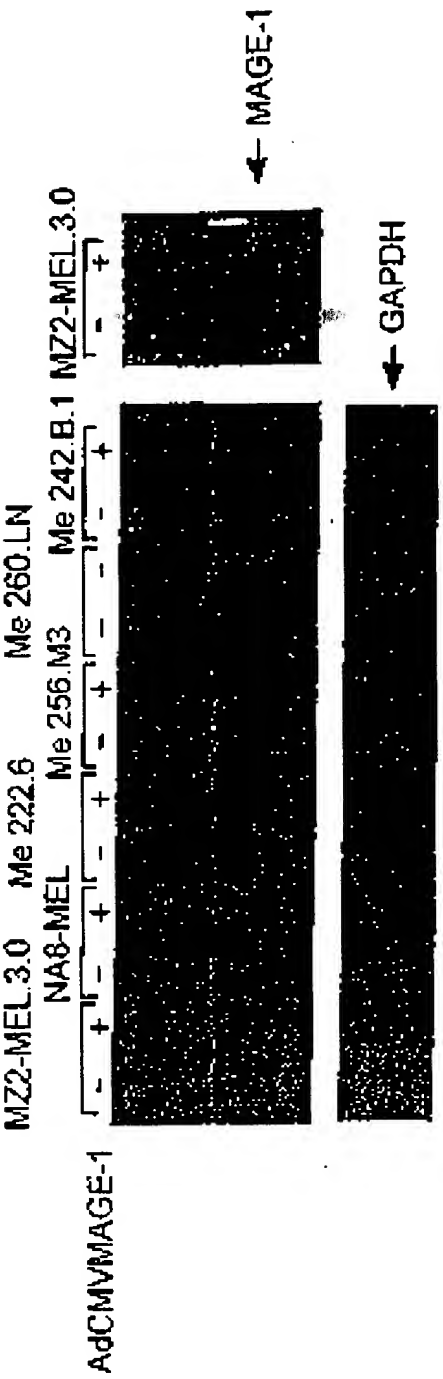


FIG. 4A

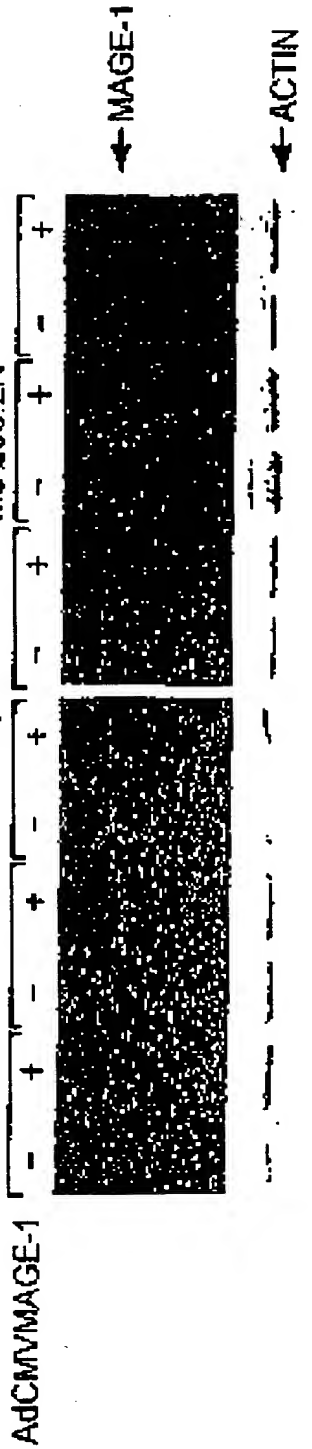


FIG. 4B

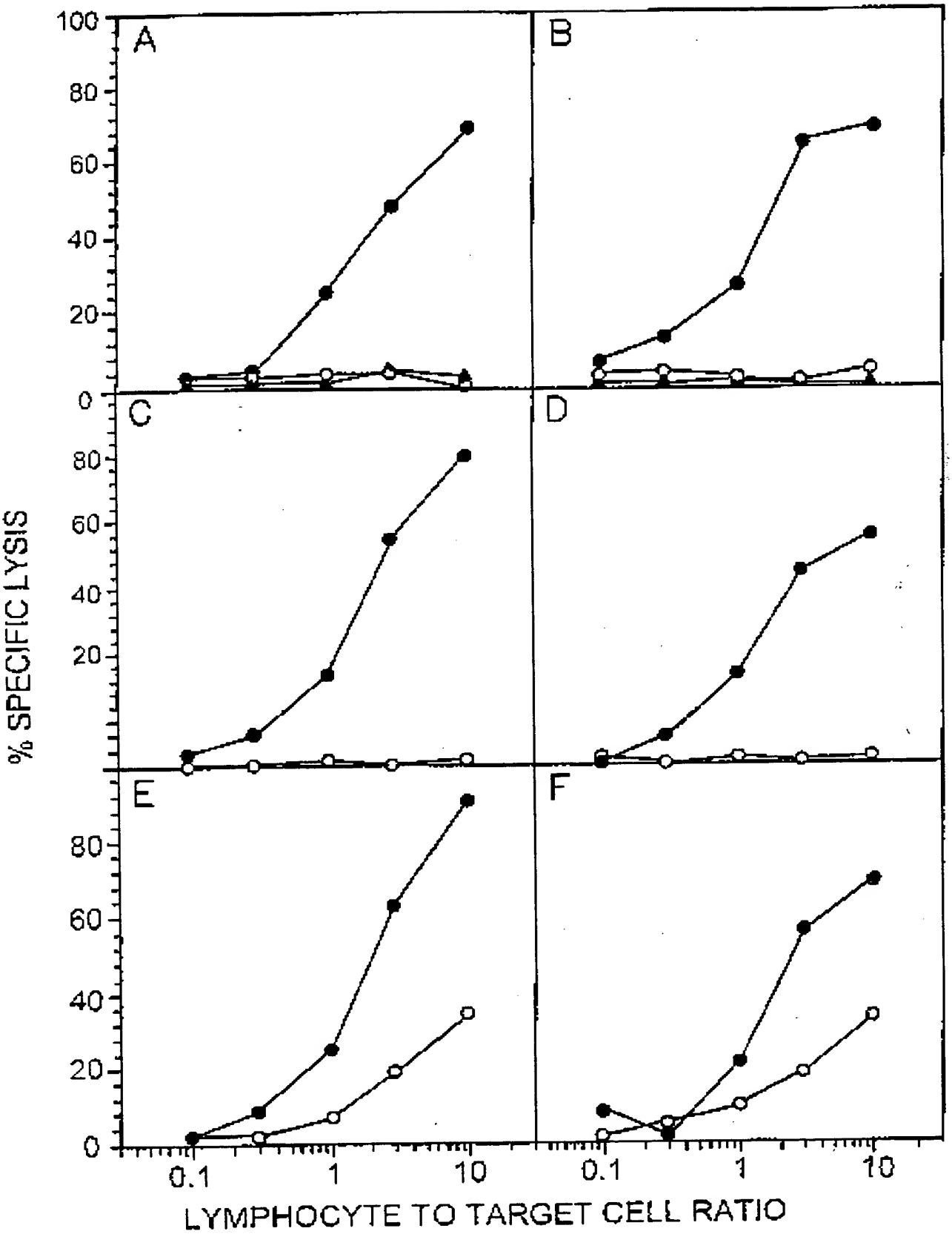


Fig. 5

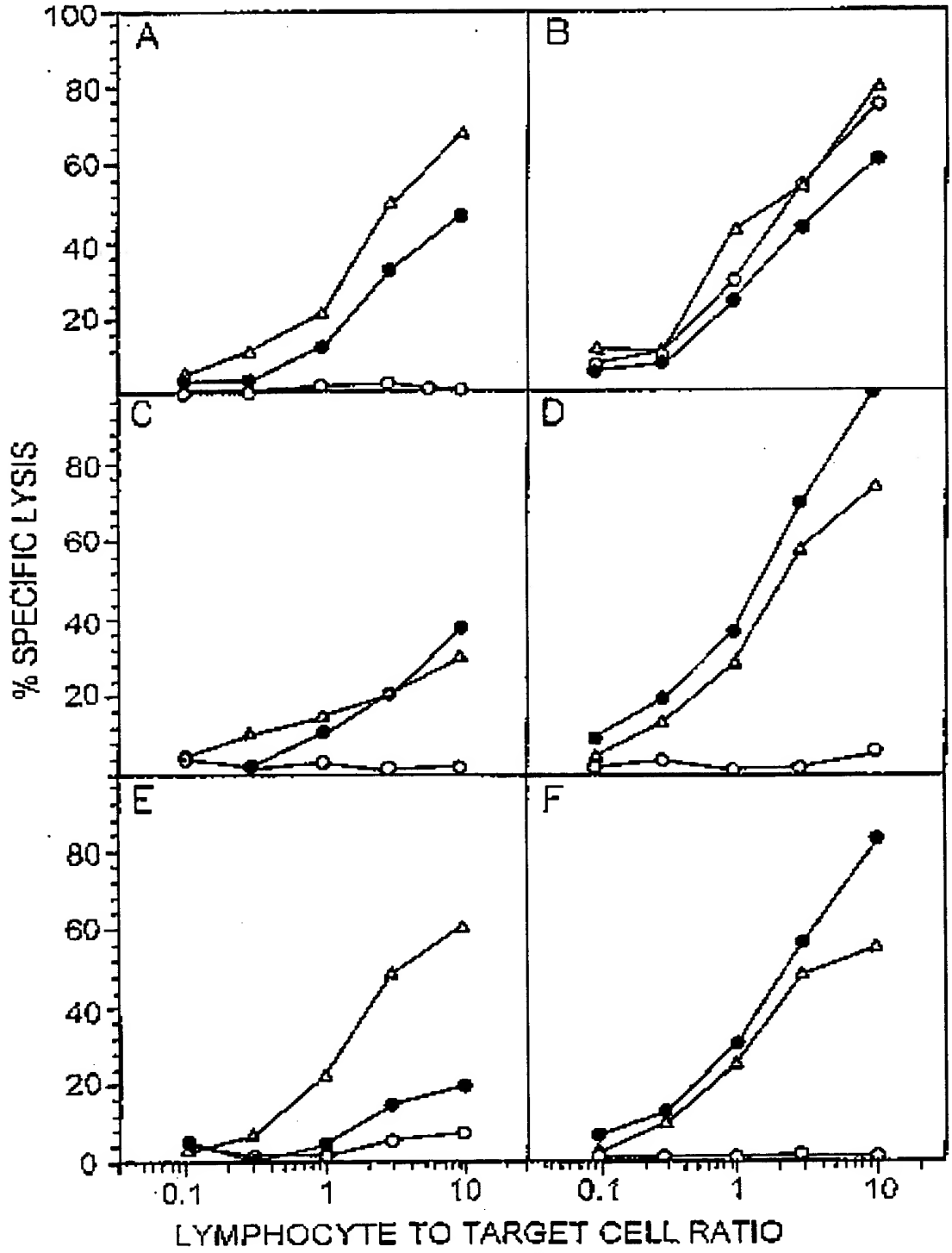


Fig. 6

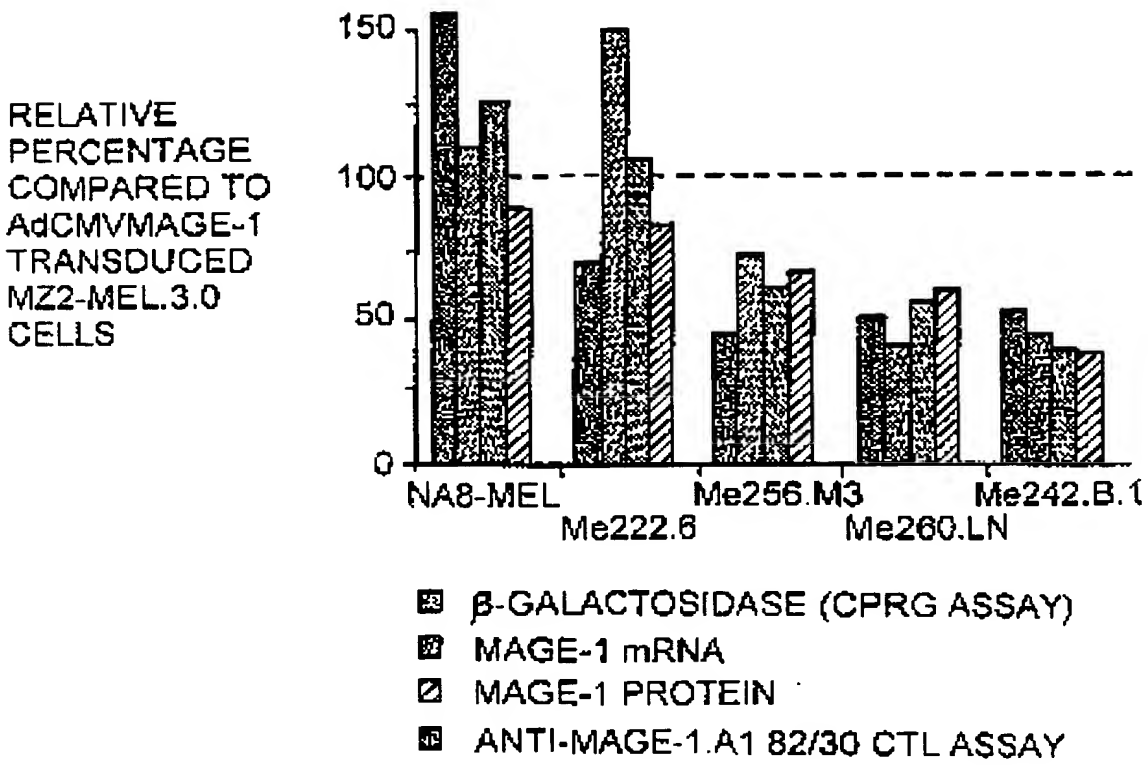


Fig. 7

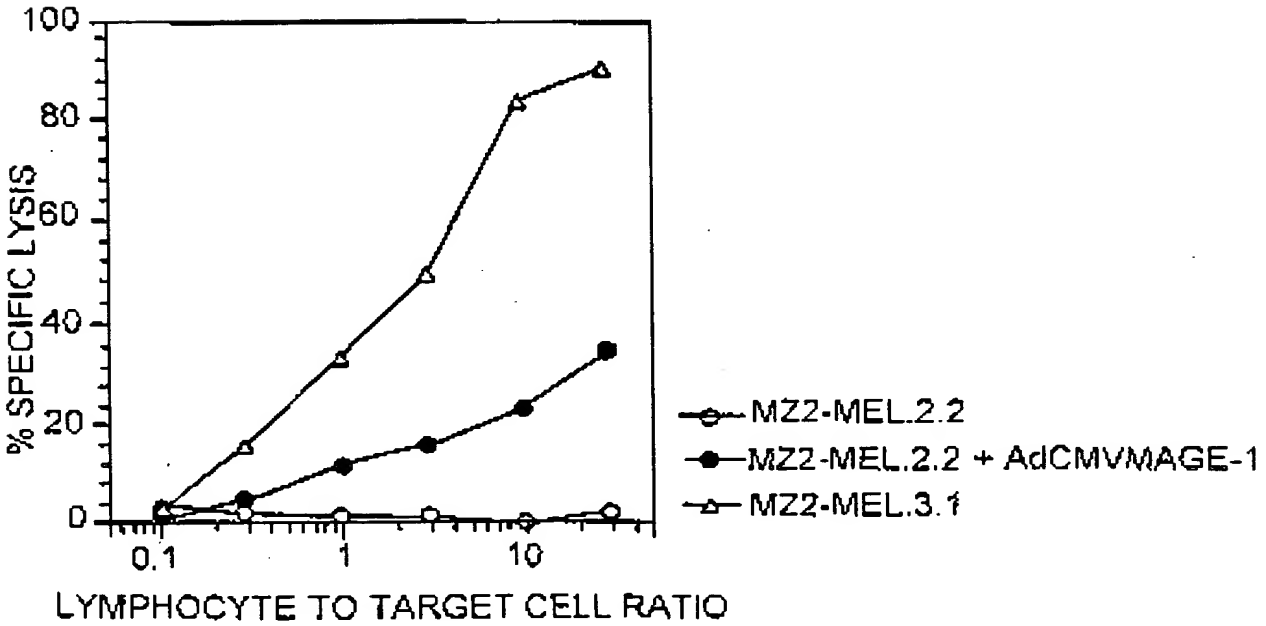


FIG. 8A

STIMULATOR CELL	TARGET CELL (BM 21)																											
MZ2-MEL.3.1	ALONE	1	1	3	1	1	3	3	4	1	1	0	2	0	1	2	0	2	0	1	1	0	0	1	0	2	2
	+MAGE-1 161-169	11	8	6	14	5	14	36	32	13	7	15	29	10	7	22	14	35	5	22	22	23	7	12	9	26	5
MZ2-MEL.2.2 INFECTED WITH AdCMVMAGE-1	ALONE	0	0	2	1	0	1	1	1	0	0																
	+MAGE-1 161-169	8	10	13	6	4	35	8	38	23	10																

FIG. 8B

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
C12N 15/86, 15/12, C07K 14/705, A61K 48/00	A3	WO 98/15638
		(43) International Publicati n Date: 16 April 1998 (16.04.98)

(21) International Application Number: PCT/US97/17948

(22) International Filing Date: 6 October 1997 (06.10.97)

(30) Priority Data:
60/027,891 6 October 1996 (06.10.96) US

(71) Applicant: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 1345 Avenue of the Americas, New York, NY 10105 (US).

(72) Inventors: REED, Darryl, S.; Chemin des Boveresses 155, CH–1066 Epalinges (CH). ROMERO, Pedro; Chemin des Boveresses 155, CH–1066 Epalinges (CH). RIMOLDI, Donata; Chemin des Boveresses 155, CH–1066 Epalinges (CH). CERROTTINI, Jean–Charles; Chemin des Boveresses 155, CH–1066 Epalinges (CH). JONGENEEL, C., Victor; Chemin des Boveresses 155, CH–1066 Epalinges (CH).

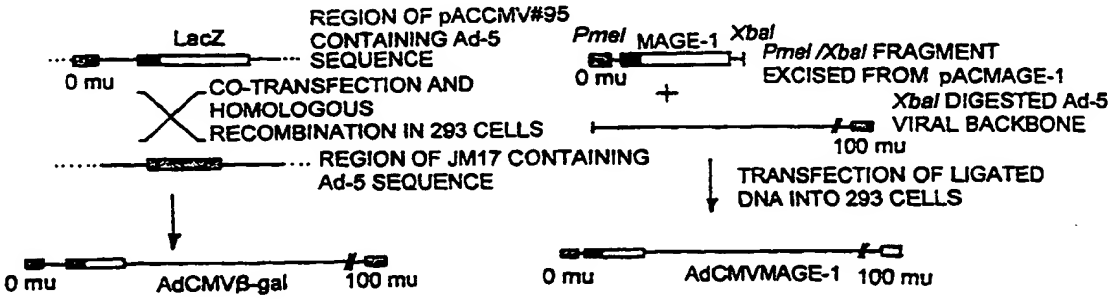
(74) Agent: VAN AMSTERDAM, John; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

(81) Designated States: AU, CA, CN, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(88) Date of publication of the international search report:
2 July 1998 (02.07.98)

(54) Title: REPLICATION–DEFECTIVE ADENOVIRUSES FOR CANCER IMMUNOTHERAPY



(57) Abstract

The invention provides compositions and methods for using replication–deflective adenoviruses and adenovirus genomes to introduce nucleic acids encoding tumor rejection antigen precursors into cells. The compositions and methods are useful for, *inter alia*, inducing or enhancing by *in vivo* or *ex vivo* means the immune response of a subject to tumor rejection antigens, preparation of cytolytic T lymphocytes and analysis of tumor rejection antigen precursor processing.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/17948

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N15/12 C07K14/705 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHAI, Y. ET AL.: "Development and characterization of recombinant adenoviruses encoding MART1 or pg100 for cancer therapy" JOURNAL OF IMMUNOLOGY, vol. 156, no. 2, 15 January 1996, pages 700-710, XP002036180 cited in the application	1-4,11, 12,14, 16, 18-20, 23-25
Y	see the whole document	5-10,15, 17,21, 22,26

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search
12 January 1998

Date of mailing of the international search report
09-04-1998

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer
Smalt, R

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 97/17948

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WARNIER G ET AL: "INDUCTION OF A CYTOLYTIC T-CELL RESPONSE IN MICE WITH A RECOMBINANT ADENOVIRUS CODING FOR TUMOR ANTIGEN P815A" INTERNATIONAL JOURNAL OF CANCER, vol. 67, no. 2, 17 July 1996, pages 303-310, XP000675638 see the whole document ---	1-5, 9-13,16, 18-20, 23-25
Y	LAUGHLIN C A ET AL: "EFFECT OF DELETIONS IN ADENOVIRUS EARLY REGION 1 GENES UPON REPLICATION OF ADENO-ASSOCIATED VIRUS" PHYSIOLOGIA PLANTARUM, vol. 41, no. 3, March 1982, pages 868-876, XP000614630 see the whole document ---	5,6
Y	BUEELER H ET AL: "INDUCTION OF ANTIGEN-SPECIFIC TUMOR IMMUNITY BY GENETIC AND CELLULAR VACCINES AGAINST MAGE: ENHANCED TUMOR PROTECTION BY COEXPRESSION OF GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR AND B7-1" MOLECULAR MEDICINE, vol. 2, no. 5, September 1996, pages 545-555, XP002035094 see abstract ---	7-10,21, 22
Y	WO 96 26742 A (UNIV CALIFORNIA ;HAMMOND H KIRK (US); GIORDANO FRANK J (US); DILLM) 6 September 1996 see page 13, line 7 - line 27 ---	15
Y	US 5 543 328 A (MCCLELLAND ALAN ET AL) 6 August 1996 see abstract ---	17
Y	KANTOFF, P. ET AL.: "Expression of human adenosine deaminase in nonhuman primates after retrovirus-mediated gene transfer" JOURNAL OF EXPERIMENTAL MEDICIN, vol. 116, 1987, pages 219-234, XP002051671 see page 232, paragraph 4 ---	26
A	PLAEN DE E ET AL: "STRUCTURE, CHROMOSOMAL LOCALIZATION, AND EXPRESSION OF 12 GENES OF THE MAGE FAMILY" IMMUNOGENETICS, vol. 40, no. 5, 1994, pages 360-369, XP000614537 see the whole document ---	7,8,21, 22

-/--

2

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 97/17948

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RALSTON R ET AL: "COMPARISON OF CELLULAR IMMUNITY TO MAGE TUMOR ANTIGENS ELICITED IN MICE USING PEPTIDES, PURIFIED PROTEIN, VACCINIA VIRUS, AND POLYNUCLEOTIDE VACCINES" JOURNAL OF CELLULAR BIOCHEMISTRY - SUPPLEMENT, 10 March 1995, page 175 XP002030629 see the whole document ---	7-10,21, 22
A	WO 94 16713 A (LUDWIG INST CANCER RES) 4 August 1994 cited in the application ---	9,10
P,X	REED, D.S. ET AL.: "Construction and characterization of a recombinant adenovirus directing expression of the MAGE-1 tumor-specific antigen" INTERNATIONAL JOURNAL OF CANCER, vol. 72, no. 6, 17 September 1997, pages 1045-1055, XP002051672 see the whole document ---	1-26
P,X	WO 97 34009 A (RHONE POULENC RORER SA ;LUDWIG INST CANCER RES (GB); BOON FALLEUR) 18 September 1997 see the whole document -----	1-5, 7-16, 18-26

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 97/ 17948

Box I Observations where certain claim w r found unsearchable (C ntinuation of it m 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 20-26 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See annex

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

8, 22 and 1-7,9-21, 23-26 (partially)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 8 and 22 completely, and 1-7, 9-21 and 23-26 partially

Replication-deficient adenoviral vector containing an insert encoding a MAGE protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

2. Claims: 1-7, 9-21 and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding a GAGE protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

3. Claims: 1-7, 9-21 and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding BAGE-1 protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

4. Claims: 1-7, 9-21 and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding RAGE-1 protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

5. Claims: 1-7, 9-21 and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding the LB33/MUM-1 protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

6. Claims: 1-7, 9-21 and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding DAGE protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

7. Claims: 1-7, 9-21 and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding NAG protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

8. Claims: 1-7, 9-21 and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding tyrosinase protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

9. Claims: 1-7, 9-21 and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding brain glycogen phosphorylase protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

10. Claims: 1-7, 9-21 and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding Melan-A protein which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

11. Claims: 1-6, 11-20, and 23-26, all partially

Replication-deficient adenoviral vector containing an insert encoding a tumour rejection antigen precursor, not covered by any of the preceding groups, which can be functionally linked to a promoter, said vector enclosed in an adenovirus coat optionally comprising targeting ligands, and a pharmaceutical preparation containing said vector or vector with coat. Method of immunization against tumour cells comprising treatment of a subject or cells isolated from a subject with said vector or vector with coat, whereby in the case of isolated cells, these cells are reintroduced into the subject.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna al Application No
PCT/US 97/17948

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9626742 A	06-09-96	AU 5028796 A	18-09-96
		AU 5457096 A	31-10-96
		CA 2188575 A	06-09-96
		EP 0760682 A	12-03-97
		JP 10501423 T	10-02-98
US 5543328 A	06-08-96	EP 0696206 A	14-02-96
		WO 9505201 A	23-02-95
WO 9416713 A	04-08-94	AU 680236 B	24-07-97
		AU 6091394 A	15-08-94
		CN 1096584 A	21-12-94
		EP 0680330 A	08-11-95
		FI 953534 A	21-07-95
		JP 8506015 T	02-07-96
		NO 952797 A	20-09-95
		US 5558995 A	24-09-96
		US 5629166 A	13-05-97
		ZA 9400407 A	26-08-94
WO 9734009 A	18-09-97	FR 2746110 A	19-09-97
		AU 2164197 A	01-10-97

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
C12N 15/86, 15/12, C07K 14/705, A61K 48/00	A3	WO 98/15638
		(43) Internati nal Publicati n Date: 16 April 1998 (16.04.98)

(21) International Application Number: PCT/US97/17948

(22) International Filing Date: 6 October 1997 (06.10.97)

(30) Priority Data:
60/027,891 6 October 1996 (06.10.96) US

(71) Applicant: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 1345 Avenue of the Americas, New York, NY 10105 (US).

(72) Inventors: REED, Darryl, S.; Chemin des Boveresses 155, CH–1066 Epalinges (CH). ROMERO, Pedro; Chemin des Boveresses 155, CH–1066 Epalinges (CH). RIMOLDI, Donata; Chemin des Boveresses 155, CH–1066 Epalinges (CH). CERROTTINI, Jean–Charles; Chemin des Boveresses 155, CH–1066 Epalinges (CH). JONGENEEL, C., Victor; Chemin des Boveresses 155, CH–1066 Epalinges (CH).

(74) Agent: VAN AMSTERDAM, John; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

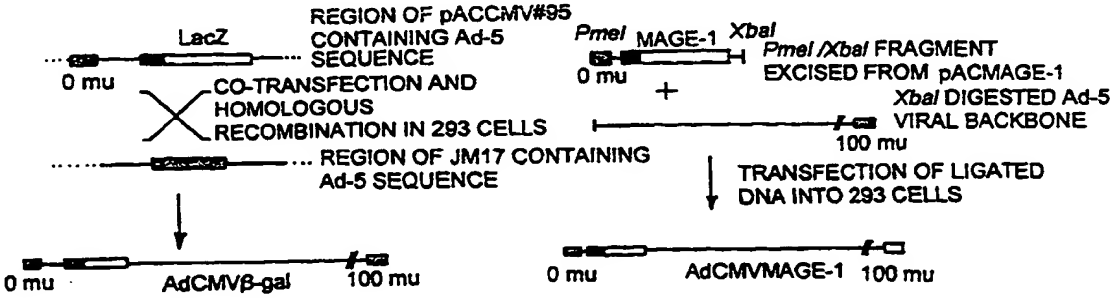
(81) Designated States: AU, CA, CN, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
With amended claims.

(88) Date of publication of the international search report:
2 July 1998 (02.07.98)

Date of publication of the amended claims:
13 August 1998 (13.08.98)

(54) Title: REPLICATION–DEFECTIVE ADENOVIRUSES FOR CANCER IMMUNOTHERAPY



(57) Abstract

The invention provides compositions and methods for using replication–deflective adenoviruses and adenovirus genomes to introduce nucleic acids encoding tumor rejection antigen precursors into cells. The compositions and methods are useful for, *inter alia*, inducing or enhancing by *in vivo* or *ex vivo* means the immune response of a subject to tumor rejection antigens, preparation of cytolytic T lymphocytes and analysis of tumor rejection antigen precursor processing.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

AMENDED CLAIMS

[received by the International Bureau on 09 June 1998 (09.06.98);
original claim 1 amended; original claims 2, 3, 5 cancelled; original claims 4-26
renumbered as claims 2-23 (4 pages)]

1. An isolated nucleic acid molecule comprising a replication-defective adenovirus genome containing an insert encoding a tumor rejection antigen precursor, wherein the adenovirus genome is an adenovirus type 2 genome or an adenovirus type 5 genome, wherein the adenovirus genome contains at least one inactivated region selected from the group consisting of E1 and E3; E1 and E4; E3 and E4; and E1, E3 and E4.

2. The isolated nucleic acid molecule of claim 1, wherein the at least one inactivated region is inactivated by deletion of a portion of the region sufficient to inactivate the at least one region.

3. The isolated nucleic acid molecule of claim 2, wherein the adenovirus genome is adenovirus type 5 deletion mutant dl309.

4. The isolated nucleic acid molecule of claim 1, wherein the insert encoding a tumor rejection antigen precursor consists essentially of a molecule selected from the group consisting of a nucleic acid molecule encoding MAGE-1, a nucleic acid molecule encoding MAGE-2, a nucleic acid molecule encoding MAGE-3, a nucleic acid molecule encoding MAGE-4, a nucleic acid molecule encoding MAGE-5, a nucleic acid molecule encoding MAGE-6, a nucleic acid molecule encoding MAGE-7, a nucleic acid molecule encoding MAGE-8, a nucleic acid molecule encoding MAGE-9, a nucleic acid molecule encoding MAGE-10, a nucleic acid molecule encoding MAGE-11, a nucleic acid molecule encoding GAGE-1, a nucleic acid molecule encoding GAGE-2, a nucleic acid molecule encoding GAGE-3, a nucleic acid molecule encoding GAGE-4, a nucleic acid molecule encoding GAGE-5, a nucleic acid molecule encoding GAGE-6, a nucleic acid molecule encoding BAGE-1, a nucleic acid molecule encoding RAGE-1, a nucleic acid molecule encoding LB33/MUM-1, a nucleic acid molecule encoding DAGE, a nucleic acid molecule encoding NAG, a nucleic acid molecule encoding MAGE-Xp2, a nucleic acid molecule encoding MAGE-Xp3, a nucleic acid molecule encoding MAGE-Xp4, a nucleic acid molecule encoding tyrosinase, a nucleic acid molecule encoding brain glycogen phosphorylase and a nucleic acid molecule encoding Melan-A.

5. The isolated nucleic acid molecule of claim 4, wherein the insert consists essentially of a

nucleic acid molecule encoding MAGE-1.

6. The isolated nucleic acid molecule of claim 4, wherein the insert consists essentially of a nucleic acid molecule encoding at least two tumor rejection antigens.

7. The isolated nucleic acid molecule of claim 4, wherein the insert consists essentially of a nucleic acid molecule encoding a tumor rejection antigen precursor capable of being processed into at least two tumor rejection antigens.

8. The isolated nucleic acid molecule of claim 1, further comprising a non-adenovirus promoter operably linked to the insert encoding the tumor rejection antigen precursor.

9. The isolated nucleic acid molecule of claim 8, wherein the promoter is non-specific with respect to tissue expression.

10. The isolated nucleic acid molecule of claim 9, wherein the promoter is selected from the group consisting of a cytomegalovirus promoter, an adenovirus E1A promoter, an adenovirus MLP promoter, a Rous sarcoma virus LTR promoter, and a SRα promoter.

11. The isolated nucleic acid molecule of claim 10, wherein the promoter is a cytomegalovirus promoter.

12. The isolated nucleic acid molecule of claim 8, wherein the promoter is selected from the group consisting of a tissue-specific promoter and a regulatable promoter.

13. A replication-defective adenovirus comprising a recombinant adenovirus genome, which consists of a nucleic acid molecule as described in any of claims 1-12, enclosed in an adenovirus coat.

14. The replication-defective adenovirus of claim 13, further comprising a targeting ligand bound to the adenovirus coat.

15. A pharmaceutical preparation comprising:
an isolated nucleic acid molecule as described in any of claims 1-12, and
a pharmaceutically acceptable carrier.

16. A pharmaceutical preparation comprising:
a replication-defective recombinant adenovirus as described in claim 13 or 14, and
a pharmaceutically acceptable carrier.

17. A method for increasing tumor-specific cytolytic T lymphocytes in a subject in need of such treatment, comprising administering to the subject a replication-defective recombinant adenovirus as described in claim 13 or 14, wherein the adenovirus genome encodes a tumor rejection antigen precursor, in an amount effective to increase cytolytic T lymphocytes specific for the tumor rejection antigen precursor or a tumor rejection antigen derived therefrom.

18. The method of claim 17, wherein the tumor rejection antigen precursor is selected from the group consisting of MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, MAGE-7, MAGE-8, MAGE-9, MAGE-10, MAGE-11, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, BAGE-1, RAGE-1, LB33/MUM-1, DAGE, NAG, MAGE-Xp2, MAGE-Xp3, MAGE-Xp4, tyrosinase, brain glycogen phosphorylase and Melan-A.

19. The method of claim 18, wherein the tumor rejection antigen precursor is MAGE-1.

20. A method for treating a subject with a disorder characterized by expression of a tumor rejection antigen precursor, comprising
administering to the subject an amount of a replication-defective adenovirus encoding the tumor rejection antigen precursor sufficient to ameliorate the disorder, the expression of the tumor rejection antigen precursor by the adenovirus increasing cytolytic T lymphocytes specific for complexes of an HLA molecule and a tumor rejection antigen that is derived from the tumor rejection antigen precursor sufficient to ameliorate the disorder.

21. The method of claim 20, further comprising administering to the subject an adjuvant.

22. A method for increasing tumor-specific cytolytic T lymphocytes in a subject in need of such treatment, comprising administering to the subject a replication-defective recombinant adenovirus genome as described in any of claims 1-12, wherein the adenovirus genome encodes a tumor rejection antigen precursor, in an amount effective to increase cytolytic T lymphocytes specific for the tumor rejection antigen precursor or a tumor rejection antigen derived therefrom.

23. The method of claim 22, wherein a cell of the subject is contacted with the replication-defective recombinant adenovirus genome *in vitro* and the cell then is administered to the subject.

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 6 : C12N 15/86, 15/12, C07K 14/705, A61K 48/00

A3

(11) International Publication Number: WO 98/15638

(43) Internati nal Publication Date: 16 April 1998 (16.04.98)

(21) International Application Number: PCT/US97/17948

(22) International Filing Date: 6 October 1997 (06.10.97)

(30) Priority Data:
60/027,891 7 October 1996 (07.10.96) US

(71) Applicant: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 1345 Avenue of the Americas, New York, NY 10105 (US).

(72) Inventors: REED, Darryl, S.; Chemin des Boveresses 155, CH–1066 Epalinges (CH). ROMERO, Pedro; Chemin des Boveresses 155, CH–1066 Epalinges (CH). RIMOLDI, Donata; Chemin des Boveresses 155, CH–1066 Epalinges (CH). CERROTTINI, Jean–Charles; Chemin des Boveresses 155, CH–1066 Epalinges (CH). JONGENEEL, C., Victor; Chemin des Boveresses 155, CH–1066 Epalinges (CH).

(74) Agent: VAN AMSTERDAM, John; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

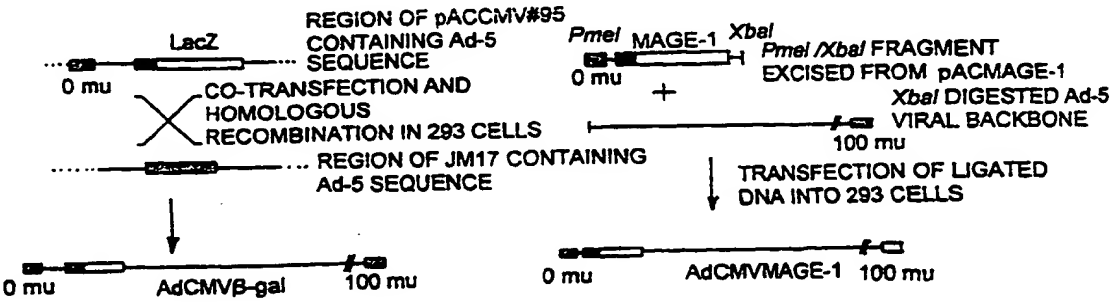
(81) Designated States: AU, CA, CN, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
With amended claims.

(88) Date of publication of the international search report:
2 July 1998 (02.07.98)

Date of publication of the amended claims:
13 August 1998 (13.08.98)

(54) Title: REPLICATION–DEFECTIVE ADENOVIRUSES FOR CANCER IMMUNOTHERAPY



(57) Abstract

The invention provides compositions and methods for using replication–deflective adenoviruses and adenovirus genomes to introduce nucleic acids encoding tumor rejection antigen precursors into cells. The compositions and methods are useful for, *inter alia*, inducing or enhancing by *in vivo* or *ex vivo* means the immune response of a subject to tumor rejection antigens, preparation of cytolytic T lymphocytes and analysis of tumor rejection antigen precursor processing.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		